A METHOD FOR THE PURE CULTIVATION OF PATHOGENIC TREPONEMA PALLIDUM (SPIROCHÆTA PALLIDA).*

By HIDEYO NOGUCHI, M.D.

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Plates 8 to 12.

Schereschewsky (1), in 1909, was the first to show that *Treponema pallidum* (*Spirochæta pallida*) might be cultivated. His method consisted in inserting a piece of human tissue containing pallida deep down into a high layer of gelatinized horse serum. In this medium, the gelatinized serum commences to liquefy about the tissue and the spirochætæ grow together with bacteria which were introduced with the inoculated material. Schereschewsky, however, never succeeded in obtaining a pure culture of the pallidum. Mühlens (2), in 1910, and Hoffmann (3), in 1911, both utilized Schereschewsky's method for obtaining first generations of the pallida and later succeeded in purifying their strains by means of Mühlens's horse serum agar. While the spirochætæ cultivated by Schereschewsky, Mühlens, and Hoffmann exhibited the typical morphological features of the pallida, these investigators, nevertheless, were unable to reproduce syphilitic lesions in animals by means of either pure or impure cultures. Subsequently, Schereschewsky stated that he had never been able to cultivate a virulent strain of the pallida, while Mühlens employed the term "pallida type" to designate his strain because it possessed no pathogenicity whatever.

On the other hand, Bruckner and Galasesco (4), in 1910, and Sowade (5), in 1911, reported that they succeeded once in reproducing syphilitic lesions in rabbits by the injection of rather large quantities of "young impure cultures" derived from gelatinized ascitic fluid or horse serum media which still contained the original syphilitic tissues. Neither investigator, however, was able to grow

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a second generation of these so-called cultures in any medium; hence the question must remain open as to whether the successful production of the syphilitic lesions was due to actually cultivated spirochætæ or simply to the original spirochætæ that had survived.

While there are few today who would deny that *Treponema pallidum* is the causative agent of syphilis, yet the final proof can only be brought forth through the reproduction of syphilitic lesions by means of pure cultures of the microörganisms. This had been done by no one up to the time of the present report.

In the present communication, I desire to report the positive reproduction of syphilitic lesions in rabbits by means of pure cultures of some of the several strains of *Treponema pallidum* which I obtained during 1910 and 1911.

MATERIALS FOR OBTAINING THE CULTURES.

Unlike all previous investigators, who used syphilitic tissues directly from human cases, I have employed the spirochætæ-containing testicular tissue of rabbits which had been inoculated with human syphilitic material for the purpose of cultivating the pallidum. The rabbit testicle, when inoculated with human material rich in pallida, swells gradually after a fortnight's incubation period and reaches its maximum, as a rule, in from four to six weeks. The testicle not only swells but its consistence becomes considerably increased through infiltration of cellular elements due to the presence of the pallida, which, at this period, reach an enormous number. From this first generation in rabbits any number of generations can subsequently be derived by transmitting the strain, at appropriate intervals, from rabbit to rabbit. The use of rabbit material offers at least two important advantages over human material; namely, (1) the ease with which large quantities of spirochæta-containing material may be obtained at any time, and (2) the fact that the spirochætæ are almost free from banal bacteria, especially after several passages through rabbits.

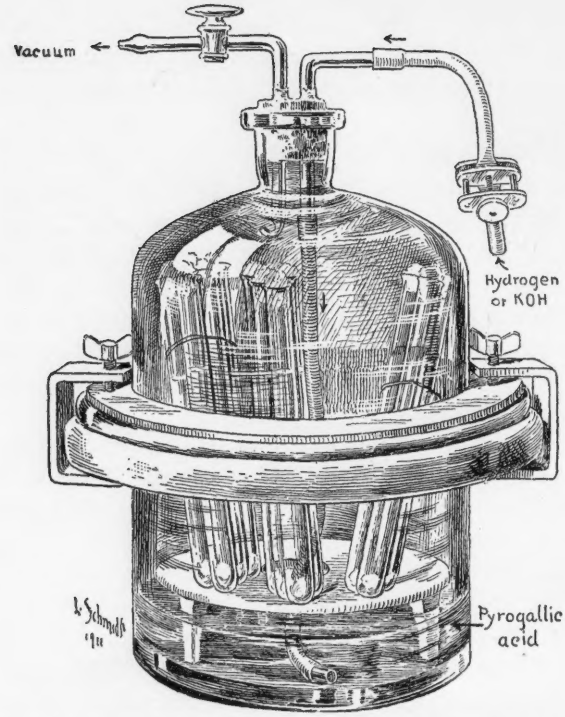
In my present work, I used ten different strains of pallida which were passed into rabbits through many generations. One of these strains was transmitted from man to the rabbit by Captain Henry

J. Nichols, of the United States Army, while working at the Rockefeller Institute; the remaining nine strains were obtained by me.

CULTURE MEDIA AND METHODS OF CULTIVATION.

Various culture media were tried, but without success. These media were pure rabbit plasma, oxalate plasma of rabbit and horse, with and without the addition of calcium chlorid (for removal of oxalate), a plasma and neutral agar mixture, a plasma and gelatin mixture, serum water, serum agar, serum bouillon to which various protein cleavage products were added (tryptophan, asparagin, glycil-glycin, alanin, etc.), and finally the gelatinized horse serum prepared according to Schereschewsky.

The only suitable medium in my hands is a serum water (sheep, horse, rabbit) to which a piece of sterile rabbit tissue is added. The rabbit tissues best adapted to this purpose are the kidney and testicle. The heart muscle is also good, but liver is unsuitable, since it is not only difficult to obtain in a sterile condition, but even when sterile, supplies enough carbohydrates to render the medium strongly acid in cases where certain bacteria are simultaneously cultivated. I usually use tubes 20 centimeters high and 1.5 centimeters wide and fill them with 16 cubic centimeters of serum water (1 part serum and 3 parts distilled water). After the completion of the usual fractional sterilization at 100° C. for three days (fifteen minutes each day), a small piece of freshly removed sterile tissue is placed in each tube. The tubes are incubated at 37° C. for two days and then examined for their sterility. To each tube a layer of sterile paraffin oil is now added in order to shield the medium from contact with the air and to prevent evaporation. With regard to the method of cultivation, it should be stated that strict anaerobic conditions are very important in obtaining the first generation of pallida. For this reason, I employ a combination of hydrogen gas, vacuum and pyrogallic acid in an anaerobic apparatus. With the fresh tissue at the bottom of the culture tube (Theobald Smith's principles for anaerobiosis) and the above apparatus, a complete anaerobic condition is obtained. There may be certain (denaturalized type) strains of pallida which grow under less



TEXT-FIGURE 1 shows the apparatus in which three conditions can be combined. The inoculated test tubes are placed inside of a jar which already contains a solution of pyrogallic acid at the bottom. Hydrogen gas is first passed from the tube indicated through the pyrogallic acid solution, for about ten minutes. After the air is driven off through the other tube (with stop-cock), the jar is sealed at both ends. The jar is then connected with a vacuum for about thirty minutes, in order to exhaust the air. When exhaustion has reached its maximum a concentrated solution of potassium hydrate is allowed to flow in from the tube indicated. Before the use of the potassium hydrate, by passing hydrogen the solution is freed from oxygen as much as possible. The potassium hydrate solution is now mixed with the pyrogallic acid solution in the jar. If there is any trace of oxygen inside the jar the mixture will quickly turn brownish or even dark brown, but if there is no oxygen it will remain almost colorless for at least many hours, when a light brown color may appear. The jar is now closed and once more hydrogen gas is passed through the mixture. Before putting the jar in the thermostat it is best to exhaust the air once more because this collapses the rubber tubing at one end, and a leak in the jar can easily be detected by the disappearance of the collapse (negative pressure) of this end. I have found it advisable to pass hydrogen gas through the inoculated culture media by means of a long, sterile capillary pipette for about five minutes before they are put in the anaerobic jar.

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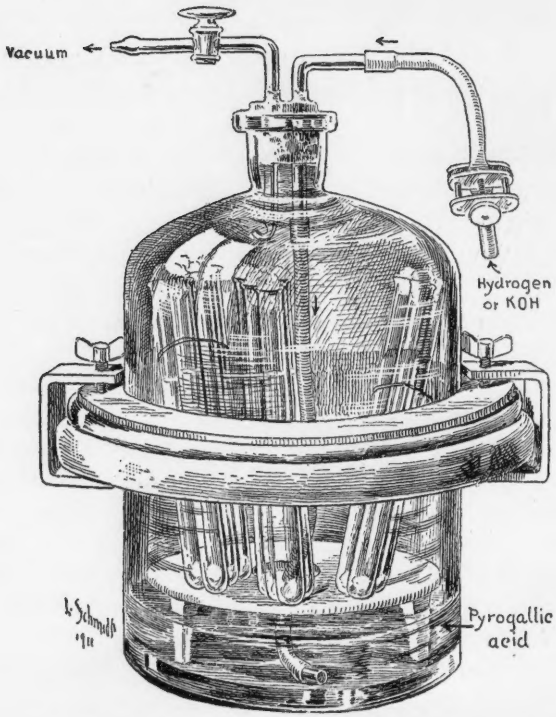
To obtain the first generation of the *Treponema pallidum* in virulent form, the following conditions are essential: (1) the presence of suitable fresh sterile tissue in serum water, (2) strict anaerobiosis, (3) a slightly alkaline reaction as furnished by the serum and tissue, and (4) a temperature of about 35° to 37° C. The presence of agar or gelatin seems to interfere with the successful growth of the first generation of the spirochætæ. On the other hand, when once adapted to the artificial serum water tissue medium, the pallidum grows well under less strictly anaerobic conditions. In fact, certain strains even grow well in a medium containing agar, provided that suitable fresh tissue is placed low down in a high column of medium (serum water or serum agar), and that it is covered with paraffin oil. Certain pallidum strains refuse to grow in a solid medium for several generations, and none of my pallidum strains grew in any medium without the presence of tissue. Thus all the pure pallidum strains are being cultivated in a tissue-containing medium. In this respect they differ greatly from those described by Mühlens and Hoffmann, since the spirochætæ of these investigators grew without difficulty in various media without tissue and even without serum.

THE RESULTS OF CULTIVATION EXPERIMENTS.

Out of ten strains of *Treponema pallidum,* six have been cultivated in the serum water tissue medium. The first strain was obtained in October, 1910, and it has just reached its twenty-fifth generation, while the remaining five strains were cultivated during the last six months. All the strains are now going on very well. It must be emphasized that to obtain these six cultures almost innumerable series of unsuccessful cultivations were carried out with each strain before one suddenly started to grow for the first time. I am still unable to explain why it is so difficult to obtain a first generation of culture with any pallidum strain; but probably the strictly parasitic nature of this organism is accountable.



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Purification.—Except in one instance, the pallidum has grown together with other bacteria. At first the pallida would not grow in any solid medium and I was unable to purify them from a fluid culture. After numerous fruitless efforts, I finally succeeded, however, in isolating the pallidum by permitting it to grow through a Berkefeld filter. Of course, only certain filters were suitable to prevent the bacteria from growing through at the same time. The spirochætæ were found to have passed through the filter after about the fifth day. Later it was found that certain strains of pallida would grow together with the bacteria along the stab canal in a serum agar tissue medium. But while the bacteria did not grow out into the surrounding medium, the pallida grew out gradually, as was indicated by a light, almost transparent zone of haziness. Thus the spirochætæ grew away from the stab canal in a pure state. From this hazy colony, I was able to obtain pure cultures from four different impure strains. The purified pallidum grows well in a serum agar tissue, but even better in a serum water tissue medium. No marked change in the culture medium is observed under anaerobic conditions. The growth does not extend very far from the bottom, where the tissue lies. I was unable, furthermore, to cultivate the spirochætæ in any medium without the addition of tissue. The mere addition of glucose to the serum agar (without tissue) does not enable the pallida to grow. When compared with the characteristics of the spirochætæ cultivated by Mühlens and Hoffmann, a striking difference is observed, since their spirochætæ grew in plain serum agar, in serum bouillon, and even in plain agar. Moreover, their strains produced a peculiar penetrating odor in serum-containing media, while my strains are odorless.

CHARACTERISTICS OF THE CULTIVATED TREPONEMA PALLIDUM.[1]

In the serum water tissue, the pallidum commences to multiply after forty-eight hours and continues to grow slowly for at least four or five weeks (probably longer). In the young cultures many

[1] The descriptions of the flagella-like projections and spore-like bodies frequently observed in the cultivated pallida are given in the explanations of the plates.

short and rather heavy specimens are seen, some with only a few curves. In a culture ten or twelve days old, the spirochætæ are of the usual length and have typical curves. As they grow older, the pallida may become very long, and often a mass of entangled pallida is formed. The cultures are quite active though probably less uniformly motile than the younger cultures. On the other hand, when cultivated in the serum agar tissue medium, the hazy zone of growth around and above the tissue becomes perceptible in about three days (sometimes two, sometimes four days). It becomes more distinct in a week or ten days. Isolated colonies are seldom formed apart from the tissue. When the inoculation is made while the medium is still in a melted state (at 45° C.), the pallida grow only around the tissue, but no individual colonies develop. It is in a stab culture only that isolated colonies appear, owing, undoubtedly, to the communication established by the stab canal between the colonies and the tissue. There is no coagulation of the serum or tissue constituents through the growth of the pallida. The morphology of the pallida cultivated in solid media is quite typical, and it is difficult to distinguish it from specimens just taken from human or animal lesions. Their motility is quite characteristic and there is no difficulty in differentiating them from *Spirochæta dentium.* In young cultures, the length of the pallidum is generally less than that of the specimens in the tissue or in an older culture, and motility is almost undiminished even after growth for some weeks. In this point they differ from the strains of Mühlens and Hoffmann, which became immobile after about ten days.

Pathogenicity.—So far only two strains of pure cultures have been carefully tested for their virulence. Both strains have produced typical lesions in the testicle of the rabbit. The lesion became noticeable about two weeks after inoculation, and the testicles were excised, under anesthesia, at the end of about four weeks for examination (dark field, and Giemsa, Levaditi, and the usual methylene blue and eosin stains). The first strain (cultivated in October, 1910, in the mixed state, and purified through a Berkefeld filter in January, 1911) was inoculated into eight rabbits on February 2, when a ten day old culture in a serum water tissue medium

was used. Two rabbits showed lesions in the course of two to five weeks. The testicles were excised under anesthesia on March 3 and showed typical syphilitic orchitis with an abundance of the pallida. The second strain (cultivated impurely in March—from the 6th to 26th—and purified in a serum agar tissue medium on April 16, 1911) was inoculated into four rabbits on April 26, when a seven day old culture in a serum water tissue medium was used. Three of the four rabbits showed typical lesions in three to five weeks. The aspirated tissue juice from the swollen testicles contained an enormous number of actively motile pallida. One of the positive animals was castrated on May 24, 1911, and the histological examination revealed the appearance shown in the illustrations.

CONCLUSION.

In conclusion, it may be pointed out that this is the first time that *Treponema pallidum* of Schaudinn has been proven beyond all doubt to have been obtained in pure culture. The method of cultivation described would appear also to be suitable for obtaining indefinite generations of the microörganism. Doubtless slight modifications will adapt it to a larger number of strains and possibly to the cultivation of all strains and to still other species of treponema. Finally, it may now be accepted as established that the testicular lesions produced in rabbits by means of syphilitic materials are the result of the multiplication of the pallida and not of some associated indefinite parasite.

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EXPLANATION OF PLATES.

PLATE 8.

FIG. 1. A two weeks old colony of the pallida grown out from an impure stab culture in a serum agar tissue medium. (Translucent view.)

FIG. 2. A three weeks old pallidum colony grown out from an impure stab culture in an ascitic fluid agar medium. (A view in reflected light.)

FIG. 3. Two weeks old pallidum colonies in a pure culture grown in an ascitic fluid agar tissue medium. The colonies are situated along two stab canals.

FIGS. 4, 5, 6, and 7. *Treponema pallidum* from a ten day old pure culture in a horse serum agar tissue medium. Giemsa stain. × 1,280.

FIGS. 8 and 9. Long forms of pallidum from a pure culture. Giemsa stain, × 1,280.

FIG. 10. A section of a pallidum colony in an ascitic fluid agar tissue medium, Giemsa stain. × 1,280.

FIG. 11. A characteristic appearance of pallida under the dark-field microscope. From a ten day old pure culture grown in an ascitic fluid agar tissue medium. The peculiar radiating arrangement of the pallida is due to the effort of each pallidum to liberate itself from the entangled mass and it can be observed within about five minutes after the slide is prepared. The mass breaks up gradually and the constituent motile pallida scatter singly or in smaller groups. (Semi-schematic.)

PLATE 9.

FIGS. 12, 13, 14, and 15. A three weeks old impure culture of pallida in a sheep serum water tissue medium. The figures show the extraordinary morphological conditions of the organism. Figure 13 shows a mass of entangled pallida. Giemsa stain. × 1,280.

FIGS. 16 and 17. A ten day old impure culture in a similar medium. Giemsa stain. ×1,280.

FIG. 18. A seven day old impure culture, showing many short forms. Giemsa stain. ×1,280.

FIG. 19. A delicate, typical form from a two weeks old impure fluid culture. Giemsa stain. × 1,280.

FIGS. 20 and 21. Pure cultures of pallida in a sheep serum water tissue medium (ten days old). Giemsa stain. × 1,280.

PLATE 10.

FIGS. 22 to 34. From impure pallida cultures in a sheep serum water tissue medium (twenty-five days old). The figures show that in spite of the enormous length of the pallida, the curves are typical. Of special interest may be the ring formation shown in figure 23. Figure 34 shows a peculiar short, but acute, curvature. Burri's Indian ink preparation. × 1,280.

PLATE 11.

FIGS. 35 to 39. Specimens of pallida from experimental syphilitic orchitis of the rabbit. For comparison with the cultivated pallidum. Giemsa stain. × 1,280.

FIG. 40. The same stained by Burri's method. × 1,280.

FIG. 41. A mass of entangled pallida derived from the syphilitic testicle of the rabbit. The preparation is made from an emulsion kept for 24 hours at 37° C. Giemsa stain. × 1,280.

FIG. 42. A section of liver of a congenitally syphilitic fetus. Levaditi method. × 1,280. (For comparison.)

FIG. 43. A section of testicle of a rabbit inoculated with the pallida from human chancre. Levaditi method. × 1,280. (For comparison.)

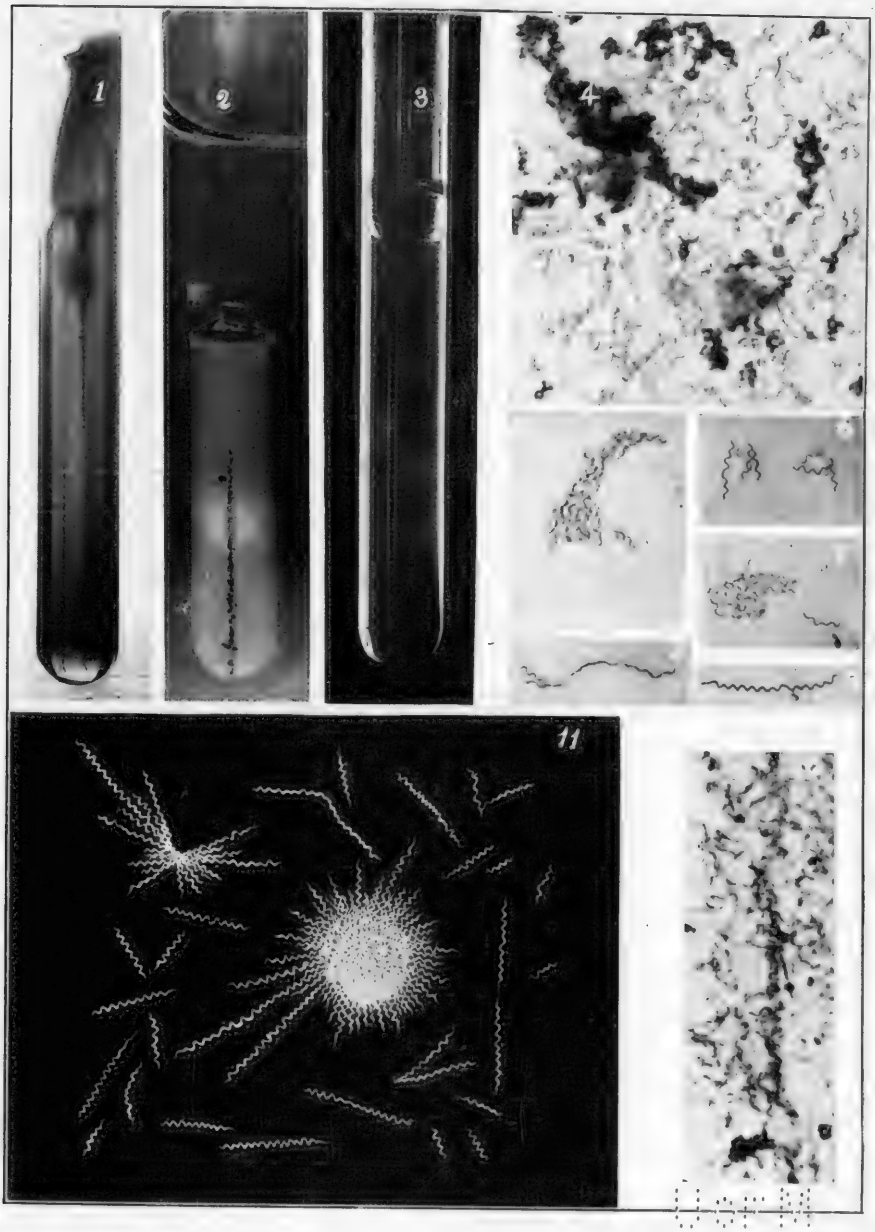
FIGS. 44 and 45. Sections of testicles of rabbits inoculated with a seven day old pure pallida culture grown in a sheep serum water tissue medium. Levaditi method. × 1,280.

PLATE 12.

FIG. 46. This figure shows several pallida with delicate flagella-like projections at one or both ends. The projections have very regular curves along their entire length, and are often seen to assume an angular position with relation to the pallida. Sometimes more than one projection may be observed at one end. It is not uncommon to see the projection attached laterally to the body of a short rod-like form of pallidum. The projections are not seen in most cultures.

Another interesting feature shown in this figure is the presence of peculiar spore-like round bodies in some pallida. Sometimes several round bodies are seen in a group, and these are entangled in a mass of short, irregular forms of pallida. It is not rare to find a round body connected with one or two young pallida as though the latter were just sprouting from the former. The pallida with these round bodies are motile. The size of this spore-like round body is variable. (Schematic; dark field.)

FIGS. 47 to 48. The round bodies mentioned above are shown here (Giemsa stain). They are difficult to stain, but when stained they take the chromatin stain. The larger ones are more or less irregular in shape and not strictly round; these seem to stain more easily than the small ones. In the right lower side of figure 47, there is one large body which shows a certain significant structure of the chromatin substance. The nature of these bodies is still unknown, but there is a striking similarity between these and a certain stage of development of *Spirochæta balbianii* studied by Perrin.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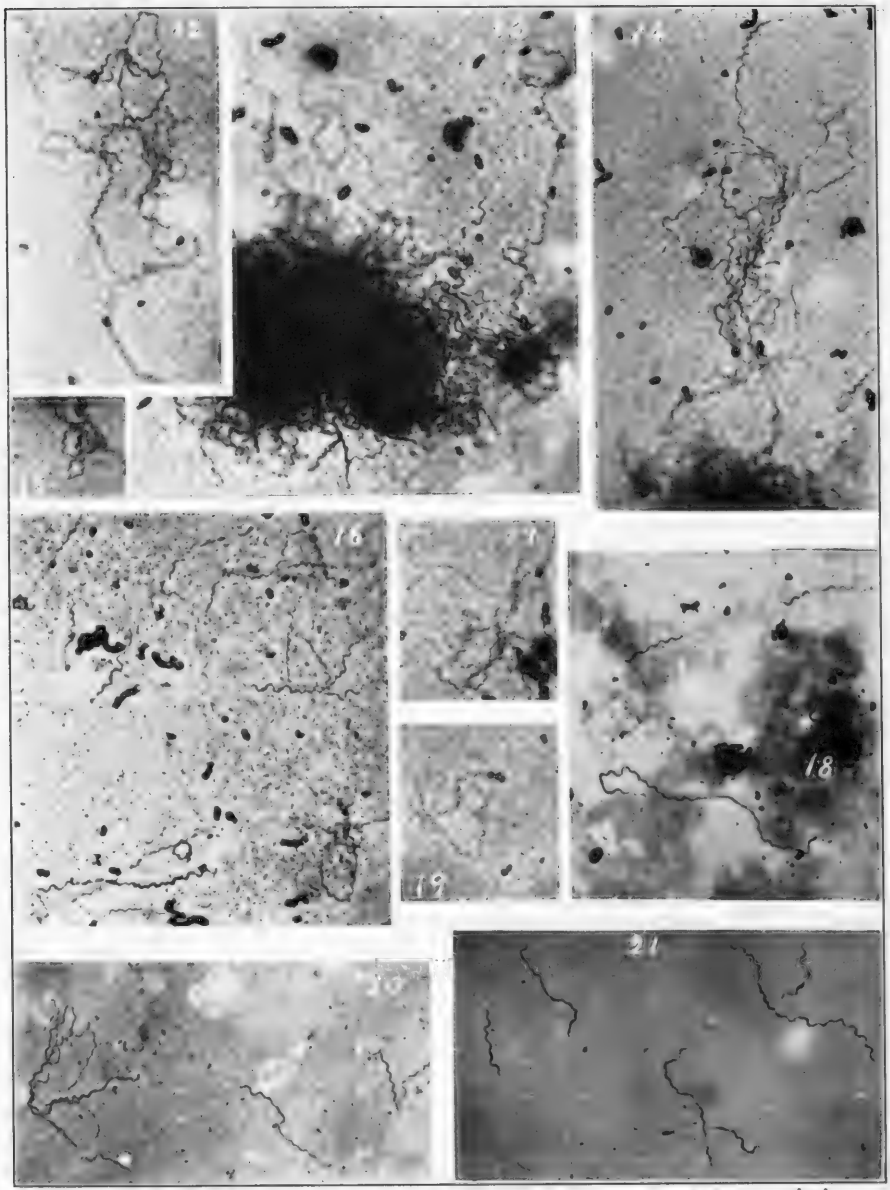
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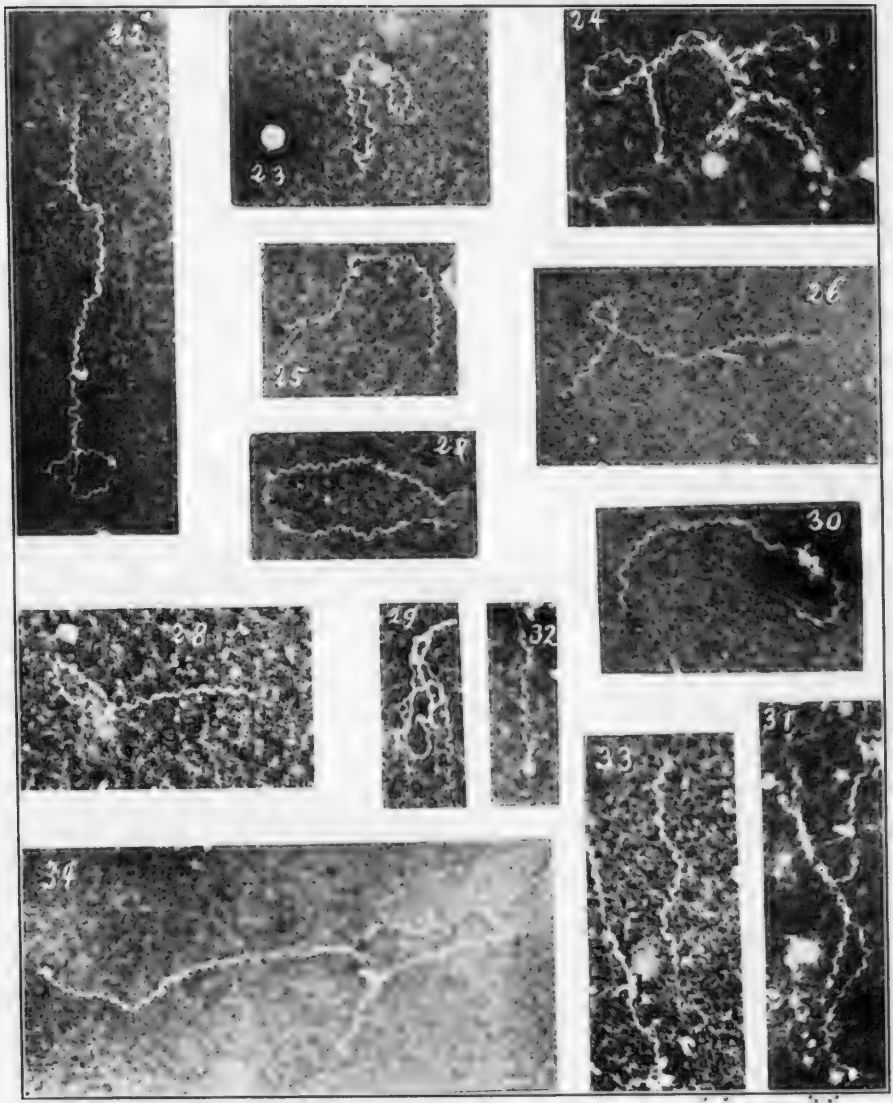
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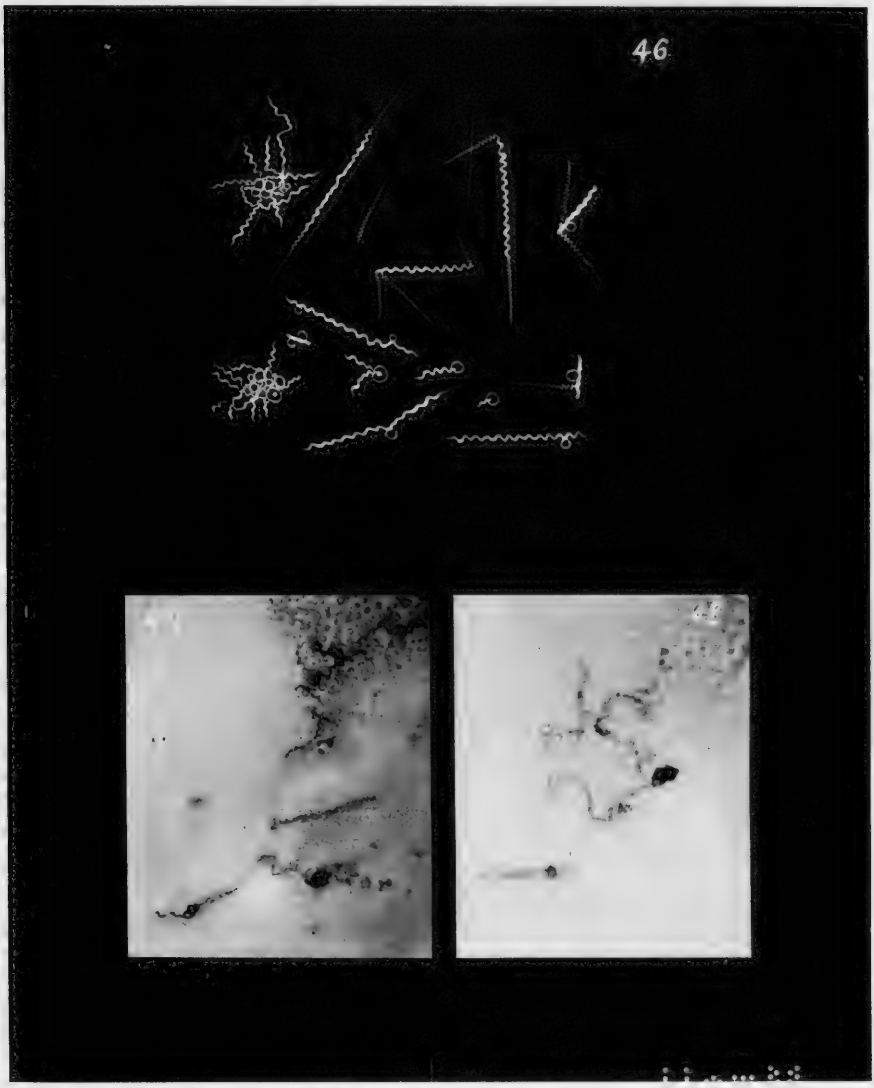
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PHAGOCYTIC IMMUNITY IN PNEUMOCOCCUS INFECTIONS, AND IN PNEUMONIA WITH RELATION TO THE CRISIS.*

By S. STROUSE, M.D.

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This work was originally planned to determine the presence of immune opsonins in the circulating blood of pneumonia patients after crisis. However, while the work was under way, Neufeld and his associates (1) published their results on the same subject, in which they claim to have found immune opsonins or bacteriotropins in practically every case. They also found that post-critical sera protected mice from many times the minimum lethal dose of virulent pneumococci. On the basis of this work, Neufeld has undertaken to make a serum for the treatment of pneumonia. Subsequently, Seligmann and Klopstock (2), and Boettcher (3) have been unable to confirm Neufeld's results.

Since Mennes' (4) first work on the subject, phagocytosis has been considered an important means of defense in pneumonia; and yet so far no successful practical application has been made of this biological reaction in treatment. Lamar (5) has recently given a conservative review of the subject of phagocytosis and pneumococcus immunity, which he concludes as follows:—

"With our present knowledge, we must view the power of an antipneumococcus serum to promote phagocytosis as the index of its therapeutic activity, and we must also view the property of spontaneous phagocytosis of the pneumococci, which certain animals exhibit, as the determining cause of their refractoriness to infection. The only known exception to this is the pigeon. . . . These considerations do not exclude the probability that there may

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be still other and undetermined factors concerned in the prevention of infection by the pneumococcus."

Seligmann (6) questions whether modern biological methods can explain the crisis in pneumonia, and Lamar has turned his attention to the chemical side of the problem.

However, in view of the importance which Neufeld attached to the presence of immune opsonins, and the inability of later workers to substantiate his findings, it seemed worth while to continue these studies. Neufeld showed in his first communications the presence of what were apparently general pneumococcus immune opsonins, but later work proved that certain strains of pneumococci were not acted upon by serum possessing opsonic powers for other pneumococci. Such strains were called "serum-fast," but they were opsonized by the post-critical sera of pneumonic patients from whom they were derived. In other words, a certain marked degree of specificity was obtained in the immune opsonins of post-critical pneumonic sera.

EXPERIMENTAL PART.

The question of the specificity of opsonic immunity in animals injected with pneumococci and the relation of such immunity to the virulence of the strain used were studied in a series of experiments. It is unnecessary to give this work in detail, as the results all coincided. With avirulent organisms it was possible to produce in the serum of injected rabbits immune opsonins for the homologous organisms, but none for heterologous strains. In all test-tube reactions, Neufeld's technique, followed precisely, was employed, as he has frequently criticized other workers on the ground that they varied the technique. In addition, however, to the test-tube experiments, the reaction was studied in the peritoneal cavity of rabbits and guinea pigs immunized to avirulent pneumococci by introducing virulent organisms and removing the peritoneal exudate by glass pipettes at different intervals. As this method of testing for immune bodies may interfere with the demonstration of a protective immunity, these experiments were always performed in duplicate. With each attempt to demonstrate phagocytic immunity in the peritoneal cavity, an exactly identical control experiment

was made without removing peritoneal fluid. In spite of the fact that the animals withstood large doses of the avirulent culture, no phagocytosis of the virulent pneumococci could be detected in the peritoneal cavity, nor were the animals rendered resistant to virulent cultures. One of these experiments is given in the following protocol:

January 23, 1911.—Guinea pigs 1–10 and 2–10. Have been immunized since December 29, 1910, with increasing doses of avirulent pneumococci. Last injection January 18, 1911.

January 23, 1911.—Guinea pig 1–10. Received 1 c.c. of virulent pneumococci intraperitoneally. Peritoneal fluid removed every 2 hours showed no phagocytosis. Pneumococci increased in number. Death in 36 hours.

Guinea pig 2–10. Same dose. No withdrawal of fluid from peritoneal cavity. Death in 20 hours.

Guinea pig X (normal control). Received intraperitoneal injection of 0.5 c.c. of the same culture. Death in 60 hours.

Perhaps the most interesting results were obtained with organisms of a moderate degree of virulence. Here the technical difficulties of estimating immune opsonins on the basis of spontaneous phagocytosis in salt solution were more easily overcome, and the attempts at immunizing were much simpler than with extremely virulent pneumococci. In this series, carried out in a parallel manner, a high degree of opsonic immunity for the homologous organism was produced. Likewise, to a certain degree, phagocytic immunity could be shown to exist for virulent strains. With some strains of pneumococci which showed slight spontaneous phagocytosis and which were only moderately virulent for rabbits, the ability to produce opsonins against virulent strains and to protect from injections of such strains was in marked contrast to the inability of avirulent organisms to produce immunity toward heterologous strains.

With very virulent organisms, there was some difficulty in raising the animals to a sufficiently high grade of immunity to withstand large doses, and in none of them could we demonstrate immune opsonins either toward the homologous virulent strain or toward heterologous avirulent or moderately virulent ones. Boettcher was likewise unable to produce phagocytic immunity for very virulent pneumococci; but he succeeded in producing in rabbits immune

opsonins for moderately virulent organisms. He also claims to have protected mice against moderately virulent organisms by previous injection of avirulent pneumococci.

These results emphasize strongly the fact that pneumococcic immunity is, to a high degree, specific for the organism used in immunization, and further that the reaction of the injected animal depends to a great extent on the virulence of the organism. My results with certain moderately virulent organisms indicate the possibility of producing in larger animals a more general immunity. Rosenow's (7) studies on the increased production of antibodies by autolyzed and washed virulent pneumococci, as compared to the difficulty of immunizing with the whole culture, may bear a close relation to the point under discussion, since, as Rosenow (8) has previously shown, virulent non-phagocytable pneumococci after autolysis become avirulent and phagocytable.

In the study of post-critical pneumonic sera, Neufeld's technique was followed. The sera were taken from twenty different patients, two to four days after crisis or lysis, and heated for twenty minutes at 54° C. The pneumococci obtained either from the sputum or blood of pneumonic patients were very virulent, were fatal in doses of 0.000001 cubic centimeters to mice, and showed no spontaneous phagocytosis. They were grown in 5 per cent. beef serum bouillon. The leucocytes were obtained from the peritoneal cavity of guinea pigs following aleuronat injection. Incubation at 37° C. was usually for one and a half hours, sometimes for two and a half hours. These are the conditions laid down by Neufeld as necessary for the successful demonstration of immune opsonins. One other factor of importance was considered in each experiment; namely, the source of the pneumococcus. If "serum-fast" strains exist, opsonizable only by the homologous serum, then each strain used must be tested against its homologous serum. This was carefully controlled in each set of experiments by using several sera and at least two organisms, including one strain homologous to one serum. A protocol of a typical experiment follows.

Our results were surprisingly constant. With the virulent organisms showing no spontaneous phagocytosis, we were never able to find opsonins in heated sera taken after the crisis from pneu-

All Sera Heated to 54° C. for Twenty Minutes. Dilutions 1:10, 1:100, 1:500, 1:1000. Incubation at 37° C. for One and a Half Hours.

Patient.	Pneumococcus.	Result.	Patient.	Pneumococcus.	Result.
A	B	No phagocytosis	A	C	No phagocytosis
B	B	No phagocytosis	B	C	No phagocytosis
C	B	No phagocytosis	C	C	No phagocytosis
D	B	No phagocytosis	D	C	No phagocytosis
E	B	No phagocytosis	E	C	No phagocytosis

Pneumococcus B is from the sputum of Patient B. Pneumococcus C is from the sputum of Patient C.

monic patients. This general result includes also those cases in which sera were tested against homologous organisms.

In addition to the experiments with highly virulent organisms, identical experiments were performed with pneumococci showing various degrees of spontaneous phagocytosis. In work of this sort, it is hard to overcome technical difficulties sufficiently to yield unbiassed and absolute readings. Great care must be taken in striking a balance between the phagocytability of the organism and the time of incubation; there is danger of the "salt control" showing too much phagocytosis, and of the attempt to overcome this by shortening the time of incubation, in which case the serum is not permitted to opsonize the pneumococci.

The plan of Lamar (9) to sensitize the pneumococci by the sera for an hour before adding the leucocytes was followed in many experiments, and at times made the results less difficult to interpret. But even with this technique, most of the work with organisms showing spontaneous phagocytosis could not be accepted by an impartial observer, and only a few experiments gave results sufficiently sharply defined to permit of conclusions. With five sera, two of which had been tested against the more virulent pneumococci with negative results, we were able definitely to show immune opsonins at a dilution of 1:300. One case of pneumococcus endocarditis receiving autogenous vaccines showed immune opsonins in a dilution of 1:1500, but to the homologous organism only. It is interesting to compare this with my other results and with those of Meakins (10) on streptococcus endocarditis. It may also be of importance in the final correlation between opsonins and actual

pneumococcus immunity to state that this patient died although her opsonins described an upward course.

Neufeld found that the post-critical sera which showed opsonins in test-tube experiments also protected mice from many times the minimum lethal dose of virulent organisms; and in my series, sera of eight different patients were tested in an identical manner. Two hours after intraperitoneal injection of 0.2 cubic centimeters of post-critical sera, various multiples of the minimum lethal dose (0.000001 cubic centimeters) of virulent pneumococci were injected. As might be expected from the test-tube experiments, these animal inoculations were completely negative. Boettcher also failed to find any protective action of post-critical sera for mice. It is probable that with less virulent cultures the same results would be obtained in animal experiments as were shown in the test tube; but an insufficient supply of mice prevented a more extensive investigation.

SUMMARY.

These results seem definitely to show, through animal experiments, that phagocytic immunity is to a high degree specific for the organism used in immunization, and that the amount of opsonin produced in the process depends to a great extent on the virulence of the organism. The negative results obtained with post-critical sera do not mean that opsonins may not be present (our five positive cases indicate their presence), but they emphasize strongly the fact that they are not formed to any great extent. Therefore this study adds further support to the view that although opsonic immunity is produced in pneumonia, it is not the only means of defense possessed by the body, and by itself it cannot explain the crisis.

In conclusion, I wish to acknowledge my indebtedness to Dr. J. W. Jobling for many helpful criticisms and suggestions in the course of this work, and to Drs. Johnson, Goodkind, and Edwards for the privilege of studying the cases in the medical wards of the hospital.

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INFECTION OF RABBITS WITH THE VIRUS OF POLIOMYELITIS.*

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The question whether rabbits succumb to the inoculation of the virus of epidemic poliomyelitis has been disputed. Krause and Meinicke[1] and, later, Lentz and Huntemüller[2] have claimed that rabbits may be infected with the virus, to the effects of which many succumb. On the other hand, Flexner and Lewis,[3] Landsteiner and Levaditi,[4] Römer and Joseph,[5] and Leiner and von Wiesner[6] all failed to produce effects in rabbits by inoculation of tissues of the spinal cord and brain from cases of human and of experimental (monkey) poliomyelitis.

It has now been established beyond peradventure that the monkey is regularly to be infected with the virus of poliomyelitis by means of intracerebral inoculation, with the result that an experimental disease is set up agreeing in all essential clinical and pathological effects with epidemic poliomyelitis in man. Conversely, the inoculation of the virus into the peritoneal cavity, subcutaneous tissue, blood, or other part distant from the central nervous system, is followed much less constantly by infection. The majority of the negative inoculations of the rabbit were made intracerebrally, and the remainder, with rare exception, intraperitoneally. Grown rabbits were usually employed. The inoculated rabbits remained well or succumbed to intercurrent diseases. However, Flexner and

* Received for publication, June 1, 1911.

[1] *Deutsch. med. Wchschr.*, 1909, xxxv, 1825; 1910, xxxvi, 693.

[2] *Ztsch. f. Hyg.*, 1910, lxvi, 481.

[3] *Jour. Exper. Med.*, 1910, xii, 227.

[4] *Compt. rend. Soc. de biol.*, 1909, lxvii, 787.

[5] *München. med. Wchschr.*, 1910, lvii, 2685; 1910, lvii, 229.

[6] *Wiener klin. Wchschr.*, 1909, xxii, 1698.

Clark[7] showed that the poliomyelitic virus would survive for a period of days in the subcutaneous tissues of the rabbit.

Although it has developed that by inoculating portions of the central nervous system of fatal cases of epidemic poliomyelitis into monkeys experimental poliomyelitis is readily produced, it has been found that the cerebrospinal fluid, blood, and other fluids from human cases fail to transmit the infection to these animals. The mesenteric lymph glands were shown by Flexner and Lewis,[8] in one instance, to harbor the virus.

If, now, we contrast with these facts, that are at present undisputed, the findings of Krause and Meinicke, and Lentz and Huntemüller, we are confronted with a remarkable discrepancy. These investigators state that the inoculation into rabbits of cerebrospinal fluid, blood, brain, or spleen obtained from human cases of poliomyelitis leads to the death of these animals, and the inoculation of similar tissues obtained from rabbits that have succumbed, into other rabbits, will bring about their death. The results, moreover, were determined in part by the mode of inoculation. When the injections were made into the blood or peritoneal cavity, a greater number of positive results was obtained than when they were made into the brain. Finally, it was ascertained that the effects could be produced in rabbits not only with emulsions of the organs mentioned but also with Berkefeld filtrates prepared from them.

To recapitulate: The monkey is subject to intracerebral inoculation with the central nervous system of human beings succumbing to epidemic poliomyelitis, to which it responds by developing a disease in all respects resembling the human affection. The correspondence includes the microscopical anatomy of the brain and spinal cord. The monkey is little, if at all, subject to inoculation with the cerebrospinal fluid or blood of human cases of the disease. It is claimed by those who have made the successful experiments with monkeys, that rabbits are insusceptible to inoculation with the virus of poliomyelitis, whether derived directly from human cases or indirectly from infected monkeys. Another set of investigators

[7] Verbal communication.

[8] *Jour. Am. Med. Assn.*, 1910, liv, 1140.

claims to have produced a fatal disease in rabbits by inoculating nervous tissue, cerebrospinal fluid, and other materials derived from human cases; in these animals, however, the symptoms of the human affection are not reproduced, and the characteristic histological lesions present in the nervous system of man and the monkey do not occur.

It is obvious, therefore, that, admitting the infection of rabbits to have been produced, it is difficult, if not impossible, to identify the result achieved with epidemic poliomyelitis of human beings or of the monkey. An effort was made to establish the result by retransferring the disease from rabbits to monkeys. Lentz and Huntemüller injected two monkeys with the filtrate of the brain and cord of a rabbit that had succumbed to the injection of cerebrospinal fluid obtained from a recently paralyzed human being, and Krause and Meinicke inoculated two monkeys intraperitoneally with an emulsion of the brain and cord from a rabbit that had been injected from a previously treated rabbit that had succumbed. In no instance did the monkeys develop the characteristic symptoms and lesions of experimental poliomyelitis. The first two monkeys showed so-called recurring transient paralysis at intervals of from two to four weeks. One died of marasmus; the other was etherized during a paralytic attack. The central nervous system failed to show the characteristic lesions, but at most showed "marked injection of vessels, hemorrhages, degenerations and small round cell infiltration of the large ganglion cells." The other two monkeys developed symptoms of nervousness and muscular weakness on the third day after intraperitoneal injection and died on the fourth day. Meinicke[9] has alluded to the occurrence in these animals of "stasis, hemorrhage, and of diffuse and perivascular infiltration of the central nervous system." The conclusion is inevitable, therefore, that the experimental conditions thus far produced by inoculating the rabbit material into monkeys cannot be identified either with the clinical or pathological effects of experimental poliomyelitis as now known.

[9] *Deutsch. med. Wchschr.*, 1910, xxxvi, 693.

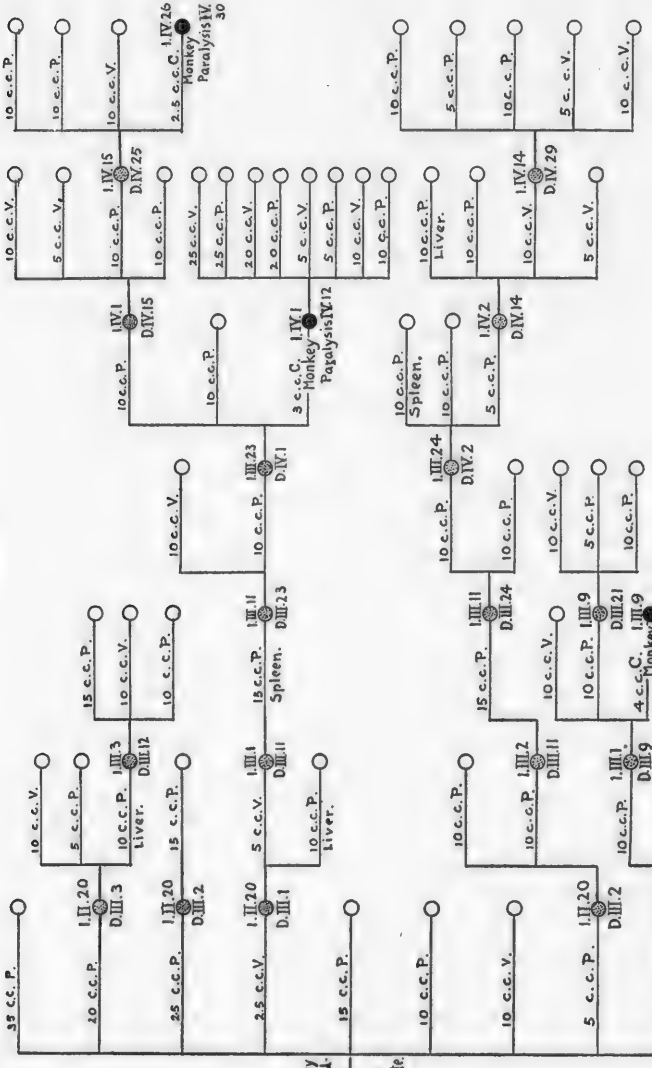
EXPERIMENTS WITH RABBITS.

The unsettled status of the problem outlined led to a review of the ground. As available samples of virus we had the M A and K material which has been under constant observation by Drs. Flexner, Lewis, and Clark for nearly two years. This virus, in the form of filtrates prepared from the central nervous system of monkeys, has not diminished perceptibly in activity and is uniformly effective by intracerebral inoculation in monkeys in doses of 0.1 cubic centimeter or less.

The effects produced in rabbits are not very definite. Death tends to occur from the seventh to the eleventh day. Just before death, motor weakness and convulsions have been noted. Our first successful experiment belongs to this class. Seventeen rabbits varying in weight between 450 and 1,250 grams were inoculated either intravenously or intraperitoneally with a monkey filtrate in doses ranging between five and twenty cubic centimeters. On the fifth day after the intraperitoneal injection of the twenty cubic centimeters of the filtrate, one rabbit, apparently perfectly well previously, suddenly developed general convulsive seizures and great motor weakness and died within an hour. The post mortem examination was negative. Cultures and film preparations were made from the blood and organs, but no bacteria were discovered, and no gross lesions of the organs were found. The sixteen rabbits left were observed over a period of two months and remained apparently well.

In repeating and continuing the experiments with rabbits, only young animals weighing from 350 to 550 grams were employed. The brain and spinal cord of a poliomyelitic monkey were mixed with saline solution in the proportion of 1 to 8, thoroughly rubbed up, shaken mechanically for half an hour, and centrifugalized, and the supernatant fluid was passed through a Berkefeld filter. Of the filtrate, from 2.5 to 35 cubic centimeters were injected intravenously or intraperitoneally into the rabbits. In carrying forward the inoculation from rabbit to rabbit, filtrates and emulsions of the brain and cord were chiefly used, but in some instances the spleen, liver, or defibrinated blood was employed. Usually the brain and cord were rubbed up with eighty cubic centimeters of saline solu-

CHART I.



I = inoculation; D = death; V = intravenous injection; P = intraperitoneal injection; C = intracerebral inoculation.
Unless otherwise specified, rabbit cord and brain were used.

tion and treated as stated above for the monkey tissue; and the spleen and liver were injected as emulsions. The results of the experiments are arranged in graphic form in Chart I.

The chief data observed may be stated as follows: There is no appreciable immediate effect produced by the injections, and nothing is noticeable for some days thereafter. When the animals succumb, death occurs between the eighth and the fifteenth day. It was not possible to determine in all instances the symptoms immediately preceding death, as this might take place during the night. When death occurred during the day the symptoms observed set in suddenly. General convulsions occurring in paroxysms, and, as a rule, tonic in character, attracted attention. The animals became more or less rigid and opisthotonic. Less often the convulsions were clonic, or both types were combined. In the interval between the seizures, more or less pronounced general weakness was evident. Death usually took place in from ten minutes to half an hour after the onset of symptoms. The post mortem examination showed merely a marked hyperemia of the cerebral cortex and no other striking condition of the nervous system or other organs. The histological examination, which was carried out most fully with the central nervous system, yielded nothing definite and characteristic. The typical perivascular infiltrative lesions of the blood vessels of the spinal cord and medulla, the degeneration of the ganglion cells, and the edema, hemorrhage, and cellular infiltration of the gray and white matter, such as are always readily seen in the human and monkey tissues, were wholly absent from the rabbit specimens.

We are convinced, however, that filtrates prepared from the nervous tissues of monkeys having experimental poliomyelitis are not wholly innocuous to young rabbits. Individual susceptibility plays a large part in this species of animal. But certain individuals succumb in a remarkable manner to the injections. Filtrates and emulsions prepared from these rabbits are capable of producing identical effects in still other rabbits. We have not succeeded in accomplishing an indefinite transfer of the experimental disease through rabbits, but we have transmitted it through a series of six rabbits. Whether this represents the limit of transfer with the

particular virus which we have used cannot be stated. It is, of course, possible that the last or seventh series contained no single susceptible individual.

However this may be, the disease thus produced in rabbits cannot be recognized as poliomyelitis. It is significant that it can be produced by Berkefeld filtrates as well as by emulsions of organs which have regularly been found devoid of bacteria. There is one way in which to determine whether these rabbits carry, even if they do not necessarily succumb to, the poliomyelitic virus; namely, to retransfer it to monkeys and thus to reproduce the typical disease and lesions in them. This effort had not been made successfully before. In our experiments, we were dealing with samples of virus that regularly produce characteristic effects in the monkey. We made three such transfers from the second, fourth, and sixth rabbit-passages, all being successful. The injections consisted of filtrates of the brain and spinal cord of the rabbits, prepared in the manner stated above. All the monkeys belonged to the *Macacus rhesus* species.

Monkey No. 1.—On March 9, inoculated intracerebrally (ether anesthesia) with 4 c.c. of the filtrate from the second rabbit passage. No symptoms until March 14, when the animal was noted to be somewhat excitable. On the next day, a tremor of the head was present. March 15, flaccid paralysis of the arms and legs appeared. March 17, death.

The post mortem and histological findings were characteristic of experimental poliomyelitis.[10]

Monkey No. 2.—On April 1, inoculated intracerebrally with 3 c.c. of the filtrate from the fourth rabbit passage. No symptoms until April 10, when excessive nervousness was noted. April 11, tremor of head present. April 12 flaccid paralysis of arms and legs. April 20, death.

The post mortem and histological findings were characteristic of experimental poliomyelitis.

Monkey No. 3.—On April 26, inoculated intracerebrally with 2.5 c.c. of the filtrate from the sixth rabbit passage. April 29, nervousness and excitability were noted. April 30, paralysis of arms and legs. May 2, death.

The post mortem and histological findings were characteristic of experimental poliomyelitis.

DISCUSSION.

The experimental results given above would, it seems, leave no doubt that the virus of poliomyelitis can be propagated in certain

[10] These findings have been described in detail by Flexner and Lewis (*Jour. Exper. Med., loc. cit.*) and will not be repeated here.

individual rabbits, at least through a limited series of inoculations. They also show that the virus is not confined to the central nervous system in these animals, but occurs, perhaps in equal amount, in other organs,—certainly in the liver and spleen. This distribution coincides with the absence of localized lesions in any organ in the rabbit, as far as has been discovered. Perhaps a finer study of the histology of the organs may reveal definite lesions of this new experimental disease. But it is certain that nothing resembling the characteristic lesions of the central nervous system in human beings and monkeys has appeared in the rabbit as a result of the inoculation of the strains of poliomyelitic virus thus far employed to infect them. Whether the virus can be adapted to the rabbit so as to bring out the special lesions of localization cannot be predicted. But it would appear that not all strains of the virus can be transmitted successfully even to a small fraction of individuals of all varieties of domesticated rabbits. In this way, probably, is to be explained the failure of Römer and Joseph[11] to secure any result whatever in their large series of tests.

There is still another aspect of the poliomyelitis question that is affected by these experimental results. It is now established that the virus of the disease can survive and probably even be propagated in a domestic animal that does not show any of the peculiar symptoms of epidemic poliomyelitis as it occurs in man. Conceivably, therefore, some animal, in relation to man, may act as a reservoir for the virus, which only occasionally and under special and peculiar circumstances transmits it to human beings. That such animal reservoirs of pathogenic microörganisms exist in nature has been conclusively proven by the establishment of the connection between infected goats and Malta fever;[12] and rendered probable in another instance, by reason of the finding that the wild antelope, without itself exhibiting obvious evidences of disease, can readily be infected with the trypanosome of sleeping sickness, from which *Glossina* may become infected.[13]

[11] *Münch. med. Wchnschr.*, 1910, lvii, 2685.

[12] Bruce, *Jour. Roy. Army Med. Corps*, 1907, viii, 225. Ref. in *Bull. de l'Inst. Pasteur*, 1907, v, 758.

[13] *Proc. Roy. Soc.*, Series B, 1911, lxxxiii, 311.

THE ULTIMATE RESULT OF A DOUBLE NEPHRECTOMY AND THE REPLANTATION OF ONE KIDNEY.*

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In order to ascertain whether an organ extirpated from and replanted in the same animal can functionate normally for a long period, I kept under observation for almost two and a half years a dog which had undergone a double nephrectomy and the replantation of one kidney. This experiment has already been mentioned.[1] In this article, I shall describe the ultimate result achieved.

Experiment.—A medium-sized young bitch, in normal health.

February 6, 1908, 10:12 A. M. Under ether anesthesia, transverse laparotomy and dissection of the left kidney. The surface of the organ is lobulated. At 10:58 A. M., two *serres fines* are applied on the renal vessels at some distance from the kidney; then the vessels are cut. The ureter is also cut at about six centimeters from the pelvis, and the kidney with its peritoneal covering is extirpated and washed with a small quantity of Locke's solution (at the temperature of the laboratory), which is injected through the renal artery. The organ is deposited in a jar of Locke's solution. Then the central ends of the renal vessels are prepared for anastomosis. The kidney is replaced in the abdominal cavity and circular suture of the artery and of the vein is performed. At 11:28 A. M., reëstablishment of the circulation and suture of the ureter. The organ is then fixed in its normal position by a suture of the peritoneum. The abdomen is closed by four planes of sutures.

4:00 P.M. Animal in excellent condition; walks and drinks.

February 7, 1908. Animal appears to be in normal condition.

February 19, 10:00 A.M. The right kidney is extirpated. Its surface is lobulated. Histological examination subsequently showed that its structure was entirely normal.

February 20. Animal has urinated abundantly. No albumen.

February 25. Animal appears normal. No albumen in urine.

March, 1909. In excellent health. Eleven pups born.

*Received for publication, June 9, 1911.

[1] Carrel, *Arch. f. klin. Chir.*, 1909, lxxxviii, 372; *Jour. Exper. Med.*, 1910, xii, 146.

December, 1909. Animal again has three pups.

May, 1910. Animal is in excellent condition.

July, 1910. The animal suddenly presented symptoms of intestinal obstruction and died. Dr. Auer had the kindness to examine the animal and to perform the autopsy. He found an ileus of the small intestine, while the kidney and other organs appeared normal. The kidney, with the vessels attached, and the adjacent parts of the aorta and vena cava, were preserved in formalin and examined in detail later.

Anatomical Examination.—The kidney is covered by normal peritoneum. Its surface is lobulated, as it was at the time of the first operation. Its position is normal and there is no evidence of the previous operation. The aorta and vena cava are normal, and on their right side is found the stump of the ligated vessels. The artery and vein of the left kidney are absolutely normal in caliber, in length, and in position. No evidence of the operation is found except a slight hardening of the connective tissue around the artery. The vessels are opened longitudinally. The caliber of the artery is normal and the circular scar of the anastomosis is easily seen. The caliber of the veins is also normal. The scar of the circular suture has completely disappeared. The ureter and pelvis are normal. The kidney does not present any evidence of sclerosis and is in all respects normal as far as the microscopical examination is concerned.

To summarize, a dog, after having undergone a double nephrectomy and the replantation of one kidney lives in excellent health for almost two and a half years. She dies of an intercurrent disease which is without relation to the operation. The autopsy shows the kidney to be normal. Hence the experiment proves definitely that the extirpation of the kidney in the dog, its perfusion with Locke's solution, the complete interruption of its circulation for fifty minutes, and the suture of its vessels and ureter do not interfere with its functions, even after a long period of time, and it indicates that from a purely surgical standpoint, the grafting of organs is a real possibility.

PATCHING OF THE ABDOMINAL AORTA WITH A PIECE OF RUBBER.*

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It has already been shown that part of an artery can be extirpated and replaced by a patch or a segment of an artery,[1] a vein, or even peritoneum[2] in a condition of active or latent life, and that the function of the repaired vessel remains normal. It has been found also that when hollow tissues, deprived of life, are grafted on an artery the vessel regenerates itself by using the dead vascular segment as a scaffold.[1] In two recent experiments, I attempted to patch an artery with inorganic and organic foreign substances in order to ascertain whether they can be used in the reparation or in the permanent intubation of large vessels.

The first experiment gave an incomplete functional result, but showed how an artery can redintegrate itself by the use of inorganic material.

Under ether anesthesia a segment of glass tubing of about five centimeters long coated with paraffin was introduced into the abdominal aorta of a dog. The pulsations of the femoral arteries remained normal. Six days after the operation, the animal was allowed to run. Paralysis of the posterior limbs developed and the pulsation of the femoral arteries disappeared. It was thought that the glass tubing had been displaced by the movements of the animal and that coagulation had occurred. Laparotomy was performed, the tube extirpated, and the aortic circulation reëstablished. The glass tubing was obliterated by a soft clot of recent formation. But it was found that the glass wall, inside of which circulation had taken place for six days, was covered by a thin, whitish membrane.

* Received for publication, June 9, 1911.

[1] Carrel, *Jour. Exper. Med.*, 1910, xii, 460.

[2] Carrel, *Jour. Exper. Med.*, 1910, xii, 139.

A tube having the appearance of a thin-walled artery could be extracted from the glass tubing. Microscopical examination showed that it was composed of a dense fibrin infiltrated by many leucocytes. It is probable that if the dog had been quiet and the glass tubing had not been displaced, the fibrinous tube would have become organized and would have formed inside of the glass tubing a new intima, in the manner of the canalization of a dissecting aneurism.

In the second experiment, a piece of the anterior wall of the abdominal aorta was resected and replaced by a piece of rubber.[3]

Experiment.—A medium-sized male dog. February 7, 1910, 9:45 A.M. under ether anesthesia, transversal laparotomy; intestines wrapped in greased silk towels and a wool blanket. 10:05 A.M. Dissection of the abdominal aorta below the renal arteries. Interruption of the circulation by two Crile forceps placed about 4 cm. from each other. Resection of a small piece of the anterior wall of the aorta. The opening is about 20 mm. long and 12 mm. wide. It is patched by a piece of thin rubber. At 10:36 A. M. the circulation is reëstablished. There is some leakage at the line of suture, which is easily controlled by a few stitches. The abdomen is closed and the operation completed by 11:00 A.M.

After the operation, there was no modification of the femoral pulse, and the animal remained in excellent health.

May 26, 1911. The femoral pulse and the aortic pulsations are normal. There is a slight thickening of the anterior wall at the level of the patching, and the artery adheres to the thoracic duct. The aortic segment bearing the rubber patch is extirpated and examined.

The lumen of the vessel is normal. There is neither stenosis nor dilatation at the level of the patch, but the wall is thickened. On the external side of the aorta there is no evidence of the patch, and the adventitia is thicker than normally. The vessel is opened by an incision made on its posterior wall. The intima is smooth and glistening. The location of the resected piece, which appears as an oblong area, neatly circumscribed, although having about the same appearance as the adjacent parts of the vessel, is easily detected. A longitudinal section shows that the piece of rubber was present in the wall. On its external side, a new adventitia had developed, while its internal side was covered by a thick layer of intimal tissue.

To summarize, the part of the abdominal aorta extirpated and

[3] This experiment was made at the suggestion of Professor Tuffier of the University of Paris.

replaced by a patch of rubber had been regenerated by the adjacent parts of the vessel. The caliber of the aorta examined fifteen mouths after the operation had not been modified, and the function had not been impaired. The experiment indicates, therefore, that a foreign inert substance, under certain conditions, does not produce an obliterative thrombosis, but can, indeed. be used in the reparation of the wall of a large artery.

THE CULTIVATION OF TISSUE IN PLASMA FROM ALIEN SPECIES.*

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PLATES 13 TO 15.

Biological differences in the body fluids of various species of animals are well recognized. Even with species so closely related zoölogically as rats and mice, there are distinguishing serological characteristics that recent studies have shown to be easily demonstrable. These differences have been interpreted as indicating the inability of a serum to supply food to alien cells. Certain phenomena in heterologous tissue transplantation have been thus explained. For example, Ribbert,[1] who found that human and guinea pig skin, when inoculated subcutaneously into rabbits, did not survive more than three days, interpreted his results on the basis of cell starvation. He believed that the foreign host could supply only salts and water to the implanted cells. Furthermore, Ehrlich's theory of athreptic immunity[2] as applied to the resistance manifested under certain conditions toward the transplantable rat and mouse tumors, has probably its strongest support in the behavior of a mouse sarcoma when transplanted to rats, and in the results of further transplantations to rats and mice of the nodules so produced. Ehrlich explains the phenomena by assuming the existence of a specific food stuff present only in the mouse, the exhaustion of which leads to the death of the tumor cells.

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A historical resumé of the development of the method of cultivating tissues *in vitro* was given in our article, "Characteristics of Growth of Sarcoma and Carcinoma Cultivated in Vitro," *Jour. Exper. Med.*, 1911, xiii, 495.

[1] Ribbert, *Verhandl. d. deutsch. path. Gesellsch.*, 1904, viii, 104.

[2] Ehrlich, *Arb. a. d. k. Inst. f. exper. Therapie zu Frankfurt*, 1906, No. 1, 77.

The most thorough study that has been made of the question of heterologous tissue transplantation is that of Leo Loeb[3] upon the transplantation of guinea pig epithelium to animals of other species. Since his findings have a direct bearing upon the problems with which we have been concerned, his work will be briefly outlined.

Small pieces of guinea pig skin were inoculated subcutaneously into guinea pigs, rabbits, dogs, pigeons, and frogs. Retransplantation of the grafts from the alien hosts to guinea pigs were made at varying intervals for testing the viability of the cells, or rather for determining the extent of the injury suffered in their sojourn in the foreign species. The duration of growth of the epithelial grafts under these conditions, as indicated by the presence of mitoses, was found to be eight days in rabbits, seven days in dogs, and five days in pigeons. In frogs, the cells appeared to survive only a few hours. In homologous transplantations there were formed, after eight to ten days, minute epithelial cysts which seem to persist indefinitely.

The cultivation of tissues outside the body appeared to us to offer an excellent method for investigating the question of food specificity within the species, the toxic or cytolytic actions of foreign sera, and possibly, also, the reaction of tissue to an unfavorable but non-toxic medium; that is, the reaction of cells to an injury not severe enough to cause death. Apart from its ease and simplicity, the method offered several advantages over procedures involving the grafting of tissue in the animal body: first, the possibility of observing continuously the changes occurring in a single piece of tissue under the altered environment; and secondly, the elimination of factors, which, in investigating the conditions that influence the fate of grafts in the body, have to be taken into account. Thus Loeb showed that variations in the susceptibility of different species to bacterial infections, infiltration of the grafts by leucocytes, and the constricting action of the reactive connective tissue stroma and capsule, all undoubtedly influenced the fate of the grafts of guinea pig epithelium.

In approaching these problems, our studies have been directed chiefly toward determining the suitability of plasma from a number of species as culture media for certain mouse and rat tissues,—

[3] Loeb, Leo, and Addison, W. H. F., *Arch. f. Entwcklngsmechn. d. Organ.*, 1909, xxvii, 73.

mouse carcinoma, rat sarcoma, and rat spleen,—with an attempt to analyze some of the phenomena observed.

TECHNIQUE.

For obtaining blood from mice, rats, guinea pigs, and rabbits, we have used the method described in a former paper,[4] that is, under anesthesia, the carotid artery is exposed, cleaned, ligated above, and clamped below. Its wall is caught by fine forceps and the vessel is severed distally. The clamp is then released and the blood allowed to spurt into paraffined tubes packed in ice. With rat blood, considerable care in avoiding contamination with tissue juices is necessary in order to prevent rapid coagulation. In this respect, the handling of mouse blood offers less difficulty; that of rabbits and guinea pigs may be handled with still greater ease. Dogs were bled from the femoral artery or vein through a needle of good caliber, previously boiled in albolene. These vessels are easily exposed by a small incision. Human blood was obtained through a needle from a superficial vein in the arm or forearm. In goats the same procedure was employed, either the jugular or the prominent superficial vein crossing the leg just above the knee being used. With pigeons, special precautions against contamination with tissue juices either by the use of a needle or by the method of direct flow from the vessel were found to be quite unnecessary. After removing the feathers and sterilizing the skin, the large wing vein was severed by a single incision through the skin and subcutaneous tissue with scissors or scalpel, and the blood allowed to drop into paraffined tubes. Plasma thus obtained remained fluid in some instances for hours when kept cool, and at no time did coagulation take place before completion of the experiments.

The remainder of the technique differs in only one or two minor points from that described by Burrows[5] for chick embryos. Small drops of plasma are placed on cover glasses, and finely divided pieces of tissue, 0.5 to 1 mm. in diameter, are added immediately. The cover glasses are then inverted over fairly deep, hollow ground slides, previously ringed with vaseline. The slide preparations are

[4] Lambert and Hanes, *Jour. Exper. Med.*, 1911, xiii, 495.

[5] Burrows, *Jour. Exper. Zool.*, 1911, x, 63.

incubated at 37.5° C. A warm, wooden microscope box is provided for subsequent observations. We have made no attempt at keeping the tissue warm during dissection, but we have studiously guarded against drying.

For determining the viability of pieces of tissue at any time during the course of an experiment, we have transferred them to homologous plasma. Since experience has shown that with proper care in the choice of tissue and in its handling, practically every piece of sarcoma, carcinoma, and spleen will grow when placed in homologous plasma, this test, besides being quick and simple, is thoroughly reliable. Animal inoculations were also made, but aside from the delay in noting the results, the procedure has other objections as a test of viability. In the first place, it is applicable only to preparations of tumors; and secondly, as we pointed out in a former paper,[6] failures may indicate simply a decreased virulence.

CULTIVATION OF TISSUE IN ALIEN PLASMA.

In a former note,[7] we recorded the fact that mouse and rat plasma could be interchanged as culture media for the tumors of these species, but that growth seemed to be more vigorous when homologous plasma was used. Plasma from guinea pigs, rabbits, dogs, goats, pigeons, and human beings has been employed in the course of the present investigation. In the succeeding paragraphs, the character of the growth observed in the use of the different kinds of plasma will be briefly described and frequent comparisons drawn from the growth observed in rat plasma. Three tissues,—rat sarcoma, mouse sarcoma, and rat spleen,—were used in nearly all of the experiments, but since they did not differ essentially in their behavior *in vitro,* description in most cases will be limited to the growth of rat sarcoma. Reference to the other tissues will be made only where the phenomena seemed of additional interest.

Guinea Pig Plasma.—As a culture medium for rat sarcoma, we have found guinea pig plasma only slightly less suitable than rat plasma, the difference consisting chiefly in the extent of the outwandering of cells. In some preparations, the cells remained viable

[6] Lambert and Hanes, *Jour. Exper. Med.,* 1911, xiii, 505.

[7] Lambert and Hanes, *Proc. Soc. Exper. Biol. and Med.,* 1911, viii, 59.

in a single drop of plasma for twelve to fifteen days, although there was apparently little growth after the seventh day. Several specimens were transferred every seven days to fresh drops of plasma, and marked activity was observed following four such transfers; that is, after a month's sojourn in the alien medium. Such preparations were not distinguishable from those of similar age in which rat plasma was used.

This observation is of more than passing interest in that it has an important bearing on the question of food specificity within the species. According to Ribbert's conception, we should be compelled to believe that these cells could maintain life for such a period upon a nutriment of salts and water plus the insignificant amount of rat serum carried over in the first transfer; or, with Ehrlich, we should have to assume that a sufficient amount of *X-stoff* was retained to support life for this time. A more reasonable conception, it seems to us, is that rat cells are able to take up all necessary food from guinea pig serum, and that the failure of rat sarcoma inoculations in guinea pigs to develop tumors is dependent on obscure and probably more complex factors. Mouse carcinoma seems to grow almost as well in guinea pig plasma as in rat plasma. Mitoses were observed after eight days, and cultures nine days old produced tumors when inoculated into mice. Figure 1 shows a three day growth of mouse carcinoma in guinea pig plasma, in which is seen the characteristic sheet-like extension of the cells, with several groups ("alveoli") in the fibrin meshwork beyond the advancing border. Four mitotic figures can be made out.

Rabbit Plasma.—The growth of both mouse and rat tumors is much less active in rabbit plasma than in guinea pig and rat plasma. Liquefaction of the fibrin is often quite marked. With sarcoma, the growth, though relatively slow, may continue for ten to twelve days. Transfers of the pieces to rat plasma result in renewed activity. Animal inoculations of four day specimens were positive.

Dog Plasma.—Preparations of rat sarcoma in dog plasma, after one or two days, present fairly diffuse radial outgrowths of clear spindle cells, which after this time undergo rapid disintegration. About the pieces, there is often noted a clear, narrow zone due to the disappearance of the fibrin. With mouse carcinoma, liquefac-

tion of fibrin is more marked and there is little or no outwandering of cells. Transfer of pieces of sarcoma to rat plasma after two, three, four, and six days in dog plasma showed that the majority of the cells do not survive more than two days. An occasional growth was observed in the four day transfers, but none in those of longer duration.

Goat Plasma.—We have not observed any of the phenomena of growth when using goat plasma. After a few hours there is seen surrounding the pieces of tissue a wide, finely granular zone, apparently the result of cell disintegration. Liquefaction of fibrin does not occur.

In order to determine the length of life of the pieces of tissue in goat plasma, they were transferred in the usual way to rat plasma after incubation for twenty-four, forty-eight, seventy-two, and ninety-six hours. Some of the twenty-four hour specimens showed a fair growth; a smaller number of those forty-eight hours old gave evidence of life. Older preparations remained inactive. Histological sections of several pieces of tissue after two days in goat plasma revealed nothing of significance,—pyknotic nuclei and cells in various stages of disintegration.

In several experiments, a culture medium was used composed of goat plasma and rat plasma in varying proportions, with controls consisting of preparations in unmodified goat plasma and in rat plasma. The two kinds of plasma were mixed as follows: (1) goat plasma and rat plasma, equal parts; (2) goat plasma two parts, rat plasma three parts; (3) goat plasma one part, rat plasma two parts. The tissue in the goat plasma remained quite inert. The specimens in rat plasma all showed active growth. Those in mixed plasma gave rise to feeble growths of two to three days duration, the more active ones being, as a rule, those with the larger proportion of rat plasma.

The phenomena observed in the foregoing experiments suggest the presence of a substance in goat plasma toxic for rat cells. It would seem that it is not of the nature of a cytolysin, in a strict usage of the term, since there is apparently no definite cell-dissolving action, but that it is rather a body whose action on the foreign cells, if sufficiently prolonged, produces a fatal injury; that is, a cytotoxin.

A series of experiments was begun to investigate further the nature of this hypothetical substance, its resistance to heat, chemical properties, etc. Several difficulties in technique, however, were encountered, which prevented the successful pursuit of these questions.

Pigeon Plasma.—Although not comparable in rate and extent of growth to the cultures of rat sarcoma in rat plasma, the best preparations that we have obtained have been in pigeon plasma. The period of latency, that is, the time elapsing after incubation before the onset of active migration, is sometimes relatively long—two to three days as contrasted with eight to twenty-four hours in homologous plasma. The extension of the cells into the fibrin clot in a regular radiating fashion gives an appearance similar to that observed in guinea pig plasma. The morphology of the cells is, however, quite different. During the first three days they are large and clear, sometimes even swollen in appearance. They are ovoid to spindle in shape and each cell presents two or more processes connecting it with other cells. The nucleus is large and the protoplasm contains scattering, fine granules of fat. After four to five days, growth generally ceases and cell disintegration begins, preceded by a marked increase in the size and number of fat droplets. We have occasionally observed well stained nuclei in specimens eight and nine days old, but the protoplasm of such cells was highly granular and presented exceedingly ragged borders. Figure 2 shows a stained preparation three days old. The characteristic morphology of the cells is not very apparent. In fact, we have not been able to preserve well in the stained specimens the striking and distinctive appearance of the cells.

Attempts at prolonging the life of the cultures by the addition of fresh plasma, or by transferring the pieces of tissue to fresh plasma, were uniformly unsuccessful. This result may be contrasted with the success of subcultures in guinea pig plasma, where sarcoma cells remained active for at least thirty days. It is conceivable that in the case of pigeons, the theories of Ehrlich and Ribbert in regard to food specificity might find ready application, although it would be easy to advance some other explanation for the phenomena observed.

Human Plasma.—The most striking and constant phenomenon noted in the use of human plasma is the progressive liquefaction of the fibrin, which is practically complete after six or seven days. In spite of the loss of framework, there is an active migration of cells along the cover glass. The cells often wander to the very edge of the medium. As a rule, they move out separately, but we have observed in several preparations of rat spleen radial outgrowths simulating the growth in homologous plasma where the fibrin network is usually well preserved. In these cells attached to the cover glass, the most interesting changes have been noted, especially in the cultivation of rat spleen. After four or five days cells of extraordinary size begin to appear. The rapidity of the increase in size is quite remarkable; a cell thirty to forty microns in diameter may reach 500 microns in seventy-two hours. Such a giant cell examined in the fresh state shows about its center a clear nuclear area frequently presenting knob-like prominences, surrounded by a highly granular zone, which in turn merges into a clear, filmy indefinite protoplasm with processes. In their entire extent, these cells measure 100 to 700 microns in diameter. When stained (figure 3), the knob-like prominences are seen to be nuclei with distinct nuclear membranes and prominent nucleoli. These nuclei are often seen in close apposition arranged about a light, hematoxylin staining, reticular or vacuolated area. When fresh, this portion of the cell has the appearance of a large, single nucleus. The granules, which are not different from those seen in the cells of ordinary size, react to all the stains for neutral fat. The majority of the granules are small and of fairly uniform size, but occasionally large droplets are seen. The arrangement of the granules about the nuclear area is quite striking. In several cells they seemed to radiate outward in straight lines. The greater part of the protoplasm is, as a rule, quite free from granules.

Rather large, multinucleated cells have been observed in the growth of sarcoma and carcinoma in rat plasma, but in no instance did they simulate those just described. Pieces of rat spleen or tumor, when transferred to rat plasma after five or six days in human plasma, showed marked activity. Giant cells of the type described above were sometimes noted after the transfer. In fact, our first ob-

servation of such cells was made in a preparation of rat sarcoma in which the tissue had been transferred to rat plasma after five days of apparent inactivity in human plasma.

This formation of giant cells evidently represents unsuccessful attempts at cell division. With a method whereby continuous observation of any cell is practicable, the possibility of formation through a fusion of small cells is easily eliminated. It is conceivable that an injury not severe enough to kill or prevent growth may, through some derangement of the cellular mechanism, induce such a monster formation. Experimental studies with protozoa afford support for such a conception. Moreover, we have found that human sera are lytic for rat corpuscles, and, as we shall point out in a later paper, a close parallelism has been shown to exist between the hemolytic and toxic action of alien sera. Furthermore, the ragged, mossy appearance of the cells in pieces of sarcoma transferred to rat plasma after five or six days' sojourn in human plasma is also indicative of a cell injury (figure 4).

In reviewing the above experiments, it is seen that goat plasma is the only one in which no growth whatever was observed. We see further that plasma from those species most closely related zoölogically to rats, that is, guinea pigs and mice, affords the best medium for the growth of rat sarcoma. This parallelism, however, does not extend through the remaining species employed. For example, the growth of rat sarcoma is more vigorous and of longer duration in pigeon plasma than in dog plasma. Again, human plasma is distinctly less toxic than goat plasma for the tissues used. The relation of normally existing lysins in alien sera to the suitability of the corresponding plasma as a culture medium for foreign cells will be discussed in a subsequent paper.

CONCLUSIONS.

1. Rat sarcoma may be cultivated in mouse plasma and guinea pig plasma, and the growth differs only in extent from that observed in rat plasma. The cells may show active wandering in guinea pig plasma after thirty days, if transferred at proper intervals to fresh medium.

2. Rabbit plasma is less suitable than that of guinea pigs and

mice for the growth of rat sarcoma; growth is slow, but it may continue for twelve days.

3. The duration of growth of rat sarcoma in dog plasma is from two to three days.

4. The duration of the growth of rat sarcoma in pigeon plasma is four to five days. Transferring the tissue to fresh pigeon plasma does not lengthen the period of activity.

5. No growth whatever is observed of mouse and rat tissues in goat plasma. Studies of the fate of the cells indicate the presence in goat serum of a substance toxic for these tissues.

6. In preparations of rat sarcoma in human plasma, liquefaction of fibrin is regularly observed. The phenomena of growth consist in an outwandering of cells along the cover glass, and, after four to six days, the formation of giant cells. Such giant cells are produced in larger number in the cultivation of rat spleen.

7. The degree of suitability of the different kinds of alien plasma used as culture media for mouse and rat tissues does not go hand in hand with the closeness of relationship of the species.

8. Rat spleen may be cultivated as readily in foreign plasma as the virulent transplantable tumors.

EXPLANATION OF PLATES.

Plate 13.

Fig. 1. Mouse carcinoma after three days growth in guinea pig plasma. The characteristic sheet-like extension of the cells should be observed; those on the border present protoplasmic processes. At one point, the cells have grown into the fibrin meshwork in a definite alveolar fashion. Four mitotic figures are to be seen.

Fig. 2. Rat sarcoma after three days growth in pigeon plasma, showing the independent spreading of the cells regularly observed in the cultivation of sarcoma. Two mytotic figures can be seen.

Plate 14.

Fig. 3. Cells from a five day preparation of rat spleen in human plasma, stained with hematoxylin and Sudan III. Two multinuclear giant cells are seen, and two small cells of the size ordinarily observed in cultures. In the giant cells, fat granules are numerous and of rather large size.

Plate 15.

Fig. 4. Specimen of rat sarcoma transferred to rat plasma after six days sojourn in human plasma. The tissue was fixed on the fifth day of growth in homologous plasma. The spider-like appearance of the cells is shown.

FIG. 1.

FIG. 2.

Mau

FIG. 3.

Uorn

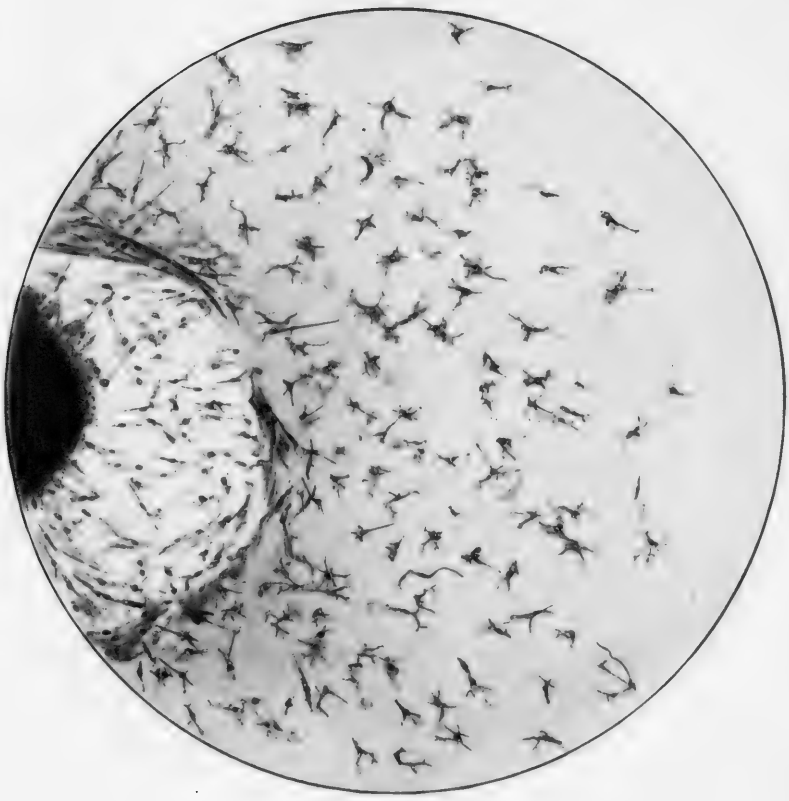

FIG. 4.

STUDIES ON IMMUNITY IN CANCERS OF THE WHITE RAT.*

The Significance of "the Specific Stroma Reaction."

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Plates 16 and 17.

The condition of immunity or resistance to the growth of an inoculable cancer is undoubtedly present in a certain percentage of animals. The mechanism of this resistance differs in many respects from the immunity of an organism against bacterial diseases. The presence of an antibody in the blood serum of a resistant animal cannot be demonstrated by any of the known methods. No irrefutable evidence is brought forward to indicate that a passive immunity may be induced by treatment of a normal animal with the blood serum of an immune one. The further fact, that unimpaired living tissue is generally required to immunize an animal against tumor growth, is the cause of the prevailing idea that immunity in cancer is due to a purely cellular activity of the organism of the host. But such hypothetical conceptions are hardly adequate to elucidate the phenomenon. Every kind of immunity represents in the final analysis the result of a cellular activity, but it is impossible to assume that the cells of a piece of fresh tissue placed under the skin and absorbed will induce an immunity in the host by their life activities. Indeed, the writer (1) has recently indicated in a series of investigations, that the same immunity may be induced by treatment with autolyzed tissue, *i. e.*, tissue in which the cells are killed but the endocellular enzymes remain active. The fact that enzymes introduced into an organism may serve to induce immunity against growth of tumor indicates that there

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must be a certain similarity between this condition and immunity in bacterial diseases. Our present methods may be inadequate to demonstrate the mechanism which is at work. The writer is engaged in the further investigation of this subject. At the same time, it seems advisable to investigate the various cellular theories of resistance against the growth of tumor. If these theories are not helpful in the further study of the subject, they must be discarded, so that further investigation of the question of resistance to cancer growth, which may prove to be of the most vital importance in the understanding of the disease, may be continued unhampered.

Ehrlich's (2) hypothesis of athrepsia, which seemed to explain readily the main phenomena of the immunity to growth of cancer, was the subject of a recent study by Levin and Sittenfield (3). The results of the investigation seem to indicate that the immunity of the rat and mouse against the growth of inoculable cancer cannot be explained merely on the basis of cellular nutrition, but tend to show an active inhibitory influence of the organism of the host on the cancer cells. Bashford (4), in his conceptions of immunity in cancers of rats and mice, denies that there is any direct influence of the organism of the host upon the inoculated cancer cell. According to his ideas, an inoculated piece of cancer tissue causes in the new host the formation of a specific connective tissue and vascular scaffolding. This connective tissue stroma surrounds the graft and furnishes it with the mechanical support and nutrition necessary for its development. Immunity then consists in the failure of the organism of the host to supply the specific stroma reaction. The immune organism does not produce a direct deleterious effect on the cells of the inoculated cancer, but alters its normal connective tissue cells so as to render them insusceptible to the chemiotactic properties of the inoculated cancer cells. Russell (5) seems to have furnished microscopic proof for this contention through his comparative microscopic studies of the "early stages" of tumor grafts in susceptible and in immune animals. In accordance with his results, there is no morphological difference noticeable in the appearance of the tissue surrounding the graft in the two kinds of animals during the first two days after a subcutaneous inoculation. But the picture changes greatly five to six days after inoculation. In the susceptible animal, there is seen an active division of the connective tissue cells of the host, which leads to the formation of a connective tissue stroma with abundant vascularisation. In the immune animal, on the other hand, there is no active proliferation of the host's fibroblasts, nor is there any development of new capillaries; the new host failing to supply a vascular stroma. Da Fano (6), in a recent investigation conducted in Bashford's laboratory, claims even to have observed a difference in the behavior of the various elements of the connective tissue of the immune host. The inoculated cancer cells retain their chemiotactic properties as against polymorphonuclear leucocytes and mast cells, but they are unable to influence the plasma cells and lymphocytes.

The question of the specificity of the reactive stroma formation after an inoculation of cancer tissue in white rats and mice, and its relation to the success of the graft, was the subject of a recent investigation by the writer (7). This study showed unmistakably that the formation of a connective tissue stroma surrounding the graft is not specific to inoculation of cancer, and is identical with the connective tissue capsule surrounding any foreign body introduced into the organism. Only when an inoculation is done subcutaneously does the transplantability of cancers of the white rat depend upon the formation of a connective tissue stroma around the implanted piece. The reason for it is to be looked for in the fact that, by the subcutaneous method, the graft is placed in a loose pocket at a distance from blood-vessels, and the prompt formation of a surrounding stroma is necessary for the support and nutrition of the inoculated cancer cells. When a piece of tumor is grafted into a parenchymatous organ, the cancer cells are placed near the blood-vessels of the organ and enabled to obtain immediately the necessary nutrition. In numerous experiments with inoculation of pieces of tumor into the brain, kidney, or testicle, there did not take place in a single instance the formation of a surrounding connective tissue stroma. The first visible step after such an inoculation into a parenchymatous organ is the proliferation of cancer cells themselves. They do not grow in a compact mass but infiltrate diffusely in all directions the parenchyma of the organ. There is no evidence of a small round cell infiltration and subsequent connective tissue stroma formation around the growing tumor. In view of these findings, it seemed of importance to investigate the behavior of both the inoculated cancer cells and the tissue of the host after an inoculation into the parenchymatous organ of an immune animal. The first question to be determined was, whether an animal rendered immune by a subcutaneous inoculation would also resist a subsequent inoculation into a parenchymatous organ. The second point for investigation was the microscopic appearance of the graft and the behavior of the neighboring tissue of the host. The present study was undertaken with these aims in view. As in the former investigation on the importance of the stroma formation, the experiments were conducted

with the same transplantable sarcoma of a white rat. Seventy-six animals previously shown to be immune against repeated subcutaneous inoculations of the tumor were used for the experiments. The rat sarcoma is a very virulent tumor, and only about 10 per cent. of the inoculated animals appear to be immune. Furthermore, since the testicle presents the best organ for the study of the microscopic appearance of the graft, the majority of the animals selected for this investigation were males. In view of all this, in order to obtain suitable animals, a selection had to be made from nearly two thousand rats which had been inoculated with the sarcoma for other purposes. Of these seventy-six animals, fifty-six, which were males, were inoculated in the testicle, and the other twenty, which were females, were inoculated in the kidney. The methods of operation were described in a previous publication (7).

These experiments showed that the tumor did not develop in a single animal; consequently an animal rendered immune by a subcutaneous inoculation of the tumor is resistant against a subsequent inoculation into a parenchymatous organ. The microscopic appearance of the graft and the surrounding tissue in the two organs are reported separately.

INOCULATION INTO THE TESTICLE.

The testicles were removed under ether anesthesia at periods of one, two, four, six, nine, twelve, and twenty-one days after the inoculation, hardened in formalin and Zenker's fluid, embedded in paraffin, and stained with hematoxylin and eosin and Mallory's anilin-blue stain. On microscopic examination, the pictures appeared strikingly different from those observed after an inoculation of the sarcoma into a testicle of a susceptible animal. The sarcoma cells of the graft preserved their morphological characteristics for the first two days only, after which they gradually degenerated. On the sixth day, one could hardly find a single well preserved cell. On the ninth day, the whole graft appeared as a homogeneous, amorphous mass, and on the twenty-first day, all traces of the graft had disappeared.

More remarkable still is the appearance of the neighboring tissue. Twenty-four hours after the inoculation, the graft was surrounded

by a round cell infiltration. On the sixth day, the mass of degenerated tumor cells was surrounded by a connective tissue stroma and by a great number of newly formed blood-vessels. This new connective tissue not only immediately surrounded the graft but spread further away from the graft between the seminiferous tubules (figure 1). In some instances, this connective tissue apparently compressed the tubules and injured the epithelial cells. Some of these tubules sustained more injury than the tubules surrounded by actively growing sarcoma in a susceptible animal. The amount of newly formed connective tissue constantly increased, and, twelve days after inoculation, in two animals the whole testicle was found filled with connective tissue spread between all the tubules (figure 2). Twenty-one days after inoculation, both the original graft and the newly formed connective tissue were found to be completely absorbed, and in most of the animals the testicle appeared to be restored to its normal condition. Occasionally a remnant of the new connective tissue was found between the tubules.

INOCULATION INTO THE KIDNEY.

The kidneys were removed under ether anesthesia at periods of one, two, four, six, fourteen, and twenty-one days, prepared, and examined in the same way as the testicles. In no instance did the tumor develop in the kidney of the immune animals. The microscopic examination showed a condition similar to the one observed in the testicles. In the first two days, some of the inoculated tumor cells were preserved, but the beginning of a connective tissue formation was noticed. Fourteen days after inoculation, the tumor cells of the graft were completely degenerated and surrounded by a stroma consisting of connective tissue and newly formed blood-vessels, which invaded the neighboring parenchyma of the kidney (figure 3). Subsequently, the graft and most of the newly formed connective tissue disappeared. The kidney, however, as a rule, was not restored entirely to its normal condition. This is probably due to the very severe operation required for the inoculation of the tumor into the kidney.

The analysis of the results of this investigation shows that Bashford's conception that immunity in cancer is due to the failure of

the tumor cell to elicit in the host a specific connective tissue and vascular stroma formation is not capable of general application. An objection may be offered that the present investigation was conducted on a rat sarcoma, while in Bashford's laboratory carcinoma of the mouse was used, and that in the former there is usually less stroma formation than in carcinoma. This difference concerns only the specific connective tissue stroma between groups of cancer cells. Bashford, Russell, and Da Fano, in describing the connective tissue and vascular scaffolding, mean primarily the layer of fibrous tissue which surrounds and encapsulates the graft. The development of this latter stroma after a subcutaneous inoculation of a rat sarcoma is identical with that of a mouse carcinoma.

The fact that cancer cells, when inoculated into a parenchymatous organ of a susceptible animal, grow without a preliminary stroma formation may not militate entirely against Bashford's conceptions of immunity, because by this method of inoculation the proliferation of the cancer cells begins so promptly that no opportunity is given for the chemiotactic properties of the cells to develop. The theory that an immune animal is an organism which resists the chemiotactic properties of the inoculated cancer cells may still be correct.

But the phenomena observed on the inoculation of cancer cells into parenchymatous organs of immune animals cannot be brought into accord with Bashford's theories. If immunity in cancer is due to the failure of the host to supply a connective tissue stroma, then the inoculated cancer cells should grow in the parenchymatous organs of the immune animal as well as in those of the susceptible one, since no preliminary stroma formation is needed for such growth. Instead of this, the immune animal kills the cancer cells inoculated in the parenchymatous organs, but, unlike the susceptible host, forms an extensive connective tissue stroma around the graft. Bashford and Russell (8), in the course of their study, made an observation which did not agree well with their theoretical considerations. This observation is as follows: When a mouse tumor is inoculated into a rat, it grows for eight or ten days and is then gradually absorbed. Through this the rat is rendered immune against a subsequent inoculation of a mouse tumor. Upon micro-

scopic examination of a mouse tumor graft inoculated into this immune rat, the authors found that after two or three days there is hardly a single cancer cell left, but that there is an extensive connective tissue stroma formed around the dying graft. They explain this phenomenon by the aid of a supposition that the immunity of a rat against the growth of a mouse tumor is not a true cancer immunity, but an active immunity against mouse tumor tissue acting as a foreign proteid. As further proof of this supposition, they state that it is possible to immunize rats against mouse tumor by previous treatment with disintegrated mouse tumor. The results of the present investigation show that the mechanism of the immunity of a rat against an inoculation of a rat tumor into an organ is identical with the immunity of a rat against a subcutaneous inoculation of a mouse tumor. In either case, the tumor cells are destroyed and surrounded subsequently by an extensive connective tissue stroma. Consequently there is produced in either case, in accordance with the conceptions of Bashford and Russell, an active immunity which leads to the rapid destruction of the cancer cell. On the other hand, it is hard to conceive of the mechanism of immunity being different solely because at one time the tumor is inoculated into a parenchymatous organ and at another subcutaneously. Goldmann (9) has also shown in a recent investigation that an immune animal does not lose the capacity for the formation of a stroma. Mice rendered completely immune against carcinoma, but not against sarcoma, produced after a subcutaneous inoculation of the latter tumor a stroma just as rich in blood-vessels as that produced by susceptible animals.

The most plausible explanation of the reactive stroma formation, and the explanation which reconciles better than any other all the known facts, is one which does not accord any specificity to the phenomenon. A cancer graft when introduced into a mouse or rat acts at first as a foreign body and as such is surrounded by the cellular elements of connective tissue. When the graft is introduced subcutaneously, the cancer cells do not become adjusted to the new host and do not begin to proliferate until a new vascular stroma is formed. On the other hand, the cancer cells have a sufficient amount of vitality to withstand the phagocytosis of the

surrounding connective tissue cells. When the graft is introduced into a parenchymatous organ, the cancer cells immediately find nutrition, begin to proliferate, and become organically united with the organ. Such a graft does not act as a foreign body and does not elicit any stroma formation. But if the same graft is inoculated into a parenchymatous organ of an immune animal, then the host, by the aid, probably, of some substance present in the body fluid, destroys the introduced cancer cells. The degenerated cells act as a foreign body and as such induce the formation of a connective tissue stroma. Ultimately both the dead cancer cells and the newly formed connective tissue are completely absorbed. Borst (10), Schmidt (11), and Orth (12) describe the same mechanism in human pathology. A connective tissue stroma surrounds a group of cancer cells, gradually increases in size, compresses the cancer cells, and produces a local cure of the growth. According to Orth, the primary factor in this condition is a degenerative change in the cancer cells, while the connective tissue stroma is of the same nature as the connective tissue which forms around a foreign body. The identical process is described by Da Fano in spontaneous healing of mouse tumors. The comparatively inefficient formation of the connective tissue stroma, after a subcutaneous inoculation of a cancer graft in an immune animal, is apparently due to the fact that the destroyed cancer cells are absorbed more rapidly from the subcutaneous tissue than from the parenchymatous organs. There is consequently less of a foreign body left to induce the stroma formation and the whole process disappears sooner.

Thus this investigation, as well as the previous studies of the writer and the study by Levin and Sittenfield, furnish further evidence to show that immunity to cancer growth is due to an active inhibitory influence of some substance present in the body fluids of the immune host, and not to any purely cellular activity. Immunity to growth of cancer must be very similar to, though not identical with, immunity in bacterial diseases.

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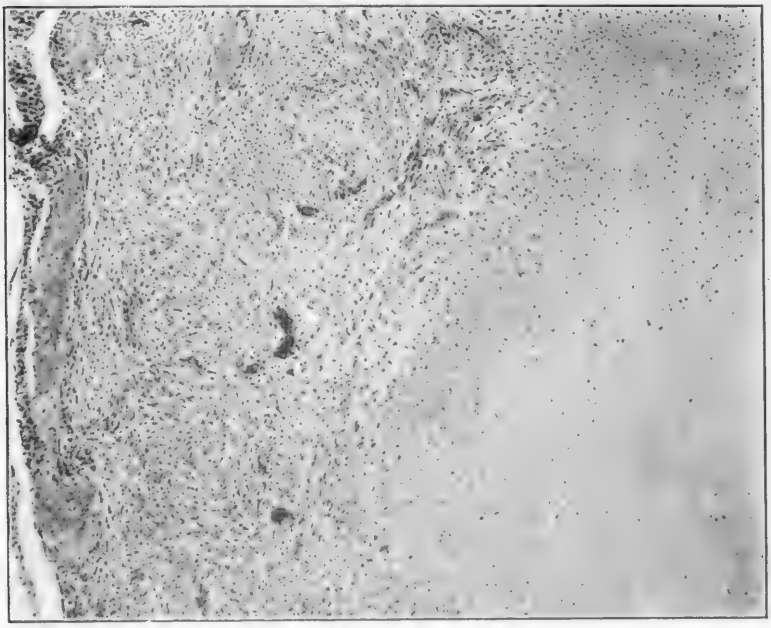

FIG. 1.

FIG. 2

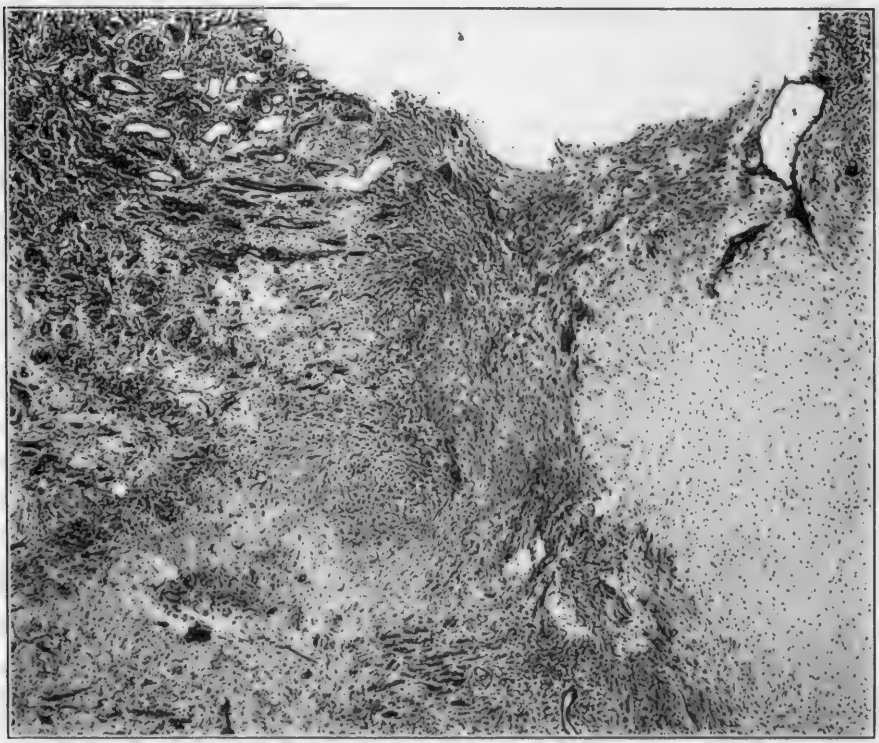

FIG. 3.

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EXPLANATION OF PLATES.

Plate 16.

Fig. 1. A tumor graft in a testicle of an immune animal. To the right of the figure is seen necrotic tumor tissue; to the left, connective tissue stroma; and further to the left, testicle tissue.

Fig. 2. A testicle of an immune rat. Newly formed connective tissue fills all the spaces between the tubules.

Plate 17.

Fig. 3. A tumor graft in a kidney of an immune animal. To the right of the figure is seen necrotic tumor tissue; further to the left, testicle tissue.

ON THE MECHANISM OF THE FORMATION OF METASTASES IN MALIGNANT TUMORS.*

AN EXPERIMENTAL STUDY.

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Plates 18 and 19.

The formation of secondary metastatic growths is characteristic of carcinoma as well as of sarcoma and presents the chief element of the malignancy of these tumors. A true metastatic growth is a secondary nodule formed in a distant organ or part of the body not contiguous to the primary tumor, and it should not be confused with even the most extensive local dissemination.

The only conceivable mode of formation of metastasis is the proliferation of a group of cancer cells, which have been transported, through the blood and lymph channels, to distant parts or organs of the body. These pieces of cancer tissue act as emboli and, finding lodgment in some parts of the organism, form secondary metastases.

Handley (1) attempted to prove recently that, at least in metastasis of cancer of the breast, embolism has no significance. According to this conception, every metastasis is formed by a process of "lymphatic permeation." In reality, the author makes use of the mechanism of the formation of local disseminations. The tumor cells grow along the lymphatic vessels until they reach the nearest lymph glands. From these glands the cells enter the next lymphatic vessel in the same manner as a fluid used for lymphatic injection, such as mercury. This process is continuous, and the appearance of an apparently isolated tumor nodule is due to the fact that a perilymphatic fibrosis destroys the permeated lymphatic vessels which form the lines of communication. Handley ascribes the formation of metastases in distant organs to the proliferation of cancer cells which escape from the subserous lymphatic plexuses into the serous cavities, pleura, or peritoneum. These cells are then distributed through these cavities under the influence of gravity and of visceral movements, and implanted on the

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serous surface of the viscera. Handley's theory is inadequate to explain all the phenomena of formation of metastasis even in cancer of the breast. Only the conception of embolic transportation of tumor cells accords with the main factors of the process both in carcinoma and sarcoma.

The main difficulty in the correct interpretation of all the phases of the formation of metastatic tumors lies in the fact that both the frequency of the occurrence of metastasis and its localization are different for the various tumors. This difference is very pronounced in carcinoma and sarcoma. Until recently it was generally accepted that the difference is due to the fact that sarcoma metastasizes through the blood-vessels and carcinoma through the lymphatics. Von Recklinghausen (2) was the first to indicate that carcinoma may also metastasize through the blood-vessels. The most thorough study, however, of the subject of the relation of blood-vessels to the formation of metastasis in carcinoma was made by Goldmann (3) and Schmidt (4). Goldmann proved that there is an extensive invasion of tumor cells into the coats of blood-vessels in carcinoma as well as in sarcoma. The paths of entrance of the cancer cells into the vascular coats appear to be the vasa vasorum, since the blood-vessel walls do not contain any lymphatics. This supposition is in accord with the fact that in arteries, the tumor cells rarely proceed further than the outer coat, whereas in veins they are generally found beneath the intima. The same difference takes place in the distribution of the nutrient vessels in the arteries and veins. While in the former the vasa vasorum usually remain within the limit of the outer coat, they extend in the veins beyond the middle coat into the region of the intima. The same investigator has shown further in his experiments with injection in the jugular vein, that the injecting fluid enters with greatest ease into the adjacent lymph glands. The conclusion must be drawn from Goldmann's investigations that there is a very intimate relation between the lymph and blood-vessels, and that carcinoma cells enter blood-vessels just as readily as sarcoma cells. Indeed, a tumor cell may penetrate a vein with greater ease than a large lymphatic vessel, since in the latter the vasa vasorum do not reach as near the inner coat as in a vein.

Schmidt examined forty-one cases of carcinoma of the abdominal viscera selected at random from his autopsy material for possible metastases in the lungs. In fifteen cases, he found, on microscopic examination, small nodules of carcinoma within the lumina of the blood-vessels of the lungs. These nodules are either surrounded by a stroma of connective tissue formed apparently by the endothelium of the blood-vessel, or else consist of a free group of cancer cells, which seem to have been transported more recently. The coats of the small pulmonary arteries, in which the cancer nodules were usually found, showed no lesion. The tumor cells apparently did not break through the wall of the blood-vessel from a focus of local dissemination, but were transported from the primary tumor or a distant metastasis. Schmidt inclines to the opinion that the carcinoma cells found in the lungs were transported through the lymphatic vessels, thoracic duct, subclavian vein, and pulmonary circulation. He finds support for this supposition in the fact that in three cases where nodules were found in the lungs there were visible metastases only in the regional lymph glands. Furthermore, he actually found small carcinoma nodules within the lumen of the thoracic duct. He does not exclude, however, the possibility that in certain cases,

in accordance with Goldmann's findings, the cancer cells may have entered a minute blood-vessel either within the primary tumor or within the metastatic tumor of the regional lymph gland, and in this way have reached the pulmonary vessel. Schmidt arrived at the conclusion that in carcinoma of abdominal organs, cancerous embolism of the small arteries of the lungs occurs with great frequency. Only a small proportion of these emboli give rise to metastatic tumors, because most of them are encapsulated and rendered harmless. A certain number of these emboli may, however, pass the pulmonary circulation and form metastases in distant organs.

This proof of the transportation of carcinoma cells through blood channels offers a better explanation for certain peculiarities in metastasis formation, than the purely lymphatic theory. In some instances, as in the formation of bone metastases in the carcinoma of the prostate, recourse had to be taken to the theory of retrograde transportation of cancer cells, to explain the formation of secondary tumors. All these investigations seem to have established firmly the fact that cells of all malignant tumors may be transported either through the lymphatics or the blood-vessels.

The difference in the channels of transportation cannot explain then the specific localization characteristic of the various malignant tumors. Different theories are offered in explanation of this phenomenon. Neusser (5) and Bamberger and Paltauf (6) believe that certain organs possess a peculiar affinity to cells of a certain malignant tumor. This selective affinity may be due to some peculiarity in the chemical constitution of the organ. Other pathologists, again, (Schmidt, Lubarsch (7), and von Recklinghausen), think that the phenomenon is caused by differences in the morphological structure of the organs. Von Recklinghausen, for instance, believes that the formation of bone metastases in carcinoma of the prostate is due to the fact that the veins and capillaries of the bone marrow have thin walls and are not collapsible. These morphological peculiarities favor the accumulation of carcinoma cells within these blood-vessels.

Lubarsch is also of the opinion that the relative size of the cancer cells and minute blood-vessels of an organ is of importance for the success of the formation of metastases.

All the investigations analyzed here were conducted on autopsy material and, as was stated above, they indicated the paths through

which the cancer cells are transported into distant parts. The real mechanism of the formation of metastases, the reason for the difference in their frequency and location for various tumors, and the reason why all cancer emboli do not form metastases, cannot be studied by mere observation on anatomical material. Only experimental investigations can throw some light on the subject. All former experiments with intravascular injection of human tumor material into animals either gave negative results or were entirely untrustworthy. The inoculable malignant tumors of white rats and mice should offer good material for the study of the subject, but there is in the literature no report of any systematic study of the mechanism of metastasis of these tumors.

In view of all this, and in view of the further fact that the study of the mechanism of the formation of metastasis may be helpful in the elucidation of many general factors in the genesis of tumors, the writers have undertaken a systematic study of the subject.

The experiments were conducted on various tumors of white rats and mice and consisted mainly of an injection of tumor emulsion into blood-vessels or joints. By the latter method, as is shown by similar experiments with bacteria, the tumor cells are introduced into the lymphatic system. A preliminary communication was published recently (8) on the methods and results of the intravascular injection of tumor emulsions. In the following report details are given of the methods of study for each tumor.

SARCOMA OF THE RAT.

This tumor is very malignant, grows locally to a large size after a subcutaneous inoculation, and usually kills the animal in about ten weeks. The formation of metastases after a subcutaneous inoculation is very rare. Though hundreds of animals were inoculated and subsequently autopsied by the writers, local dissemination was observed in not more than half a dozen cases, and once only was metastasis found in the liver.

Intravenous Injection.[1]—An emulsion of this tumor was prepared by cutting it in Haaland's mincing machine, grinding it in

[1] Ether anesthesia was employed throughout the experiments.

normal salt solution, and filtering it through a layer of coarse gauze. The milky opalescent fluid contains a sufficient number of living cells to produce a tumor growth. Control experiments with a subcutaneous injection of this emulsion showed the usual percentage of successful takes. Thirty-six rats received an injection of this emulsion into the jugular vein. The animals were killed at periods ranging from eight days to four weeks after the injection, and a thorough search for metastasis was made in all the organs. No microscopic study was made of the organs which appeared normal on gross inspection, since this investigation is not concerned with the place where the tumor cells circulating in the blood find lodgment, but with the question whether such cells transported into a certain organ will form there a visible metastasis. All suspicious nodules found anywhere and all lungs appearing abnormal on gross inspection were examined microscopically. Metastatic tumors were not found in a single animal, which is the more remarkable, since after a subcutaneous inoculation of the same emulsion about 90 per cent. of the animals grew the tumor.

Intra-Arterial Injection.—Six rats received an injection into the carotid artery, and the animals survived. This method is very difficult of execution and the animals usually die from respiratory paralysis a few minutes after the injection, though the heart continues its action a few minutes longer. No animal showed the formation of metastatic nodules.

Subcutaneous Inoculation of Tumor and Blood.—In order to test the possibility that the negative results were due to the deleterious effect of the blood on the tumor cells, a mixture was prepared of the tumor cells and defibrinated blood. The mixture was injected subcutaneously into thirty rats. Seventy-five per cent. of the animals took the tumor as against 85 per cent. in the control animals. The difference is too insignificant to indicate any influence of the blood on the tumor cells. The same subject was tested in the following series of experiments.

Inoculation into the Bone-Marrow.—The method of operation in these experiments consisted in opening the marrow of the left tibia and placing a small piece of tumor in the marrow. The opening in the bone was closed with wax and the skin sutured.

Fifteen animals were operated on in this manner, and of these, five died before an examination could be made. In eight of the remaining ten animals, a large tumor grew at the site of inoculation. But in no instance was there found any metastatic tumor. In these experiments, the inoculated cells were closely intermingled with the circulating blood, and still local tumors formed in 80 per cent. Circulating blood has consequently no appreciable deleterious effect on the tumor cells. Experiments with injection of a tumor emulsion into the joint were not done with this tumor, since a large local growth develops so promptly after an inoculation. A large tumor growth at the joint would have been so intimately connected with blood-vessels that it would have been difficult to differentiate the rôle played by the lymphatic system.

CARCINOMA OF THE RAT.

This tumor, an adenocarcinoma of the white rat described by Flexner and Jobling, seemed by its character to be more favorable for the study of metastasis than the sarcoma of the rat. The Flexner and Jobling (9) tumor possesses to a high degree the property of invading adjacent tissue, muscle, fascia, and even cartilage and bone. This tumor also frequently forms metastases after a subcutaneous inoculation. The authors described metastases in the sternum, cartilage of the ribs, in the lungs, kidney, the musculature of the heart, and in later generations even in the lymphatic glands.

Intravenous Injection.—Sixteen rats received an injection of an emulsion of this tumor into the jugular vein. In three animals, metastasis was found in the lungs (figure 1). No other organ showed any metastasis.

It must be stated here that in the intravascular injections of any of the tumors described here there has never developed a local growth at the site of the inoculation. In the experiments with the sarcoma described above, this occurred in a few animals and they were discarded as faulty experiments.

Intra-Arterial Injection.—Six rats received an injection into the carotid and survived the operation, and of these animals, one showed metastasis in the lungs. Another rat, in which the injection was made in the carotid against the stream of blood, was

found dead twelve days later. At the autopsy, a nodule was found on the wall of the left ventricle. The animal remained in the cage over night and the specimen was greatly deteriorated; still the nodule resembled microscopically the picture described by Flexner and Jobling of a metastasis found by them in the heart.

Inoculation into the Bone-Marrow.—Seven rats received the inoculation. Four animals developed a local growth in the marrow and showed no metastasis. Three animals showed no local growth and in two of these metastases were found in the lungs.

Injection into the Knee Joint.—The operation consisted in a small longitudinal incision of the skin over the knee joint and an injection of three minims of the tumor emulsion under the patella. Twenty-four rats received the injection into the joints. Eleven animals developed a small local growth in the knee joint, and in three of these, metastases were found in the lungs. Thirteen rats showed no local growth in the joint, and of these, one animal showed metastasis in the lungs.

SARCOMA OF THE MOUSE.

This tumor grows locally to a large size after a subcutaneous inoculation, but it does not form any metastases after a subcutaneous inoculation in mice. Ehrlich has shown that when this tumor is inoculated subcutaneously into a rat, a small nodule forms, which remains for eight to ten days, and is then absorbed. No metastasis formation of this mouse tumor after a subcutaneous inoculation into a rat was ever described. Experiments with intravascular injection of this tumor were performed both in mice and rats.

Intravenous Injection into Mice.—Twenty mice received an injection of an emulsion of the tumor into the jugular vein. No metastatic tumor was found anywhere in any animal.

Intravenous Injection into Rats.—Twelve rats received an injection of an emulsion of the tumor into the jugular vein. The rats were killed in periods of four to eight days, and in two of the animals metastatic nodules were found in the liver (figure 2). In no other organ was any metastasis found.

Injection into the Knee Joint of Rats.—Fifteen rats received the injection of this tumor emulsion in the knee joint. No local growth developed in the joints. One animal showed metastasis in the liver —another animal showed a very striking condition. The inguinal lymphatic glands of the opposite side to the one where the injection was made appeared suspicious on gross inspection. Microscopic examination showed that the glands were normal, but the minute blood-vessels of the fat tissue immediately surrounding the gland were completely filled with tumor cells. Figure 3 presents the condition; figure 4 is placed for comparison and shows an inguinal gland and similar blood vessels of another rat without the tumor cells. No other animal showed any metastasis anywhere.

The occurrence of metastasis in inoculable tumors of the white mouse and rat subsequent to the formation of a primary local growth is rare as compared with human cancer. The present investigation shows that it is just as difficult to induce in a healthy animal the formation of a metastatic tumor by a direct introduction of cancer cells into the lymph or blood channels. The experiments indicate further that, at least in these animals, the cancer cells, no matter how introduced, enter the blood-vessels, and through these only do they form metastases in distant organs. After an injection into blood-vessels or joints, metastases were found only in the lungs and the liver, and these organs could only be reached through the blood channels. The fact that cancer cells were found within small vessels after an injection into a joint, affords further proof for this assertion. This investigation, therefore, affords experimental proof for the supposition that metastases in malignant tumors of the rat and mouse occur through the blood-vessels. Even in the carcinoma of the rat, in which metastases were found in the lymphatic glands, Flexner and Jobling consider the blood-vessels the only paths for the formation of metastases. Carl Lewin (10) thinks that the fact that in these animals the blood-vessels are the channels for the transportation of metastases, is the reason for its rare occurrence, since the blood exerts a deleterious effect on the tumor.

The experiments with the inoculation of a mixture of tumor cells and blood, and the experiments with inoculation into the bone

marrow, indicate that direct contact of the tumor cells with blood does not impair the proliferating power of the former. It is possible, however, that the blood is capable of neutralizing the few cells which enter as emboli or are introduced as an emulsion into the circulation, but that it cannot influence the larger groups of cells introduced during an inoculation into the marrow.

Ehrlich explains the rare occurrence of metastasis in the tumors of the rat and mouse by his athreptic theory. The tumors in these animals are very malignant and grow to a large size. The cells of the primary tumor use up all the specific food found in the organism of the host, and the cells transported from the primary tumor to other regions of the organism do not find the necessary nourishment and consequently cannot proliferate. This explanation does not accord well with the facts as shown in the present investigation. It is true that the rat sarcoma gave completely negative results and that it is also the most virulent of the tumors used. None of the animals used for the experiments had any local sarcoma growth anywhere and consequently the sarcoma cells introduced into the circulation could find all the necessary specific food. No metastatic tumors, however, were formed. The most probable reason for the differences in the frequency of the occurrence of metastasis is the one which, as will be seen later, explains the differences in its localization.

The most interesting phenomenon observed in the course of these experiments is that while the Flexner-Jobling tumor found lodgment in the lungs upon intravascular injection, the mouse sarcoma, when injected into the rat, produced metastasis only in the liver. The methods of transportation were identical in each instance, and in each instance the experiments were performed on normal animals. Consequently, the differences in the morphological structure of the channels of transportation could not play any rôle. It would seem then that the most plausible explanation for the different localization of metastasis in the different kinds of malignant tumors is that it is due to a specific affinity between cancer cells and cells of certain organs. It is very interesting to note, that while the cells of the mouse sarcoma obtained lodgment, in a few instances, in the liver of the rat, they failed to produce metastasis in the mouse. As far

as it is permissible to use results obtained in experiments on lower animals for the explanation of phenomena in human pathology, it seems reasonable to suppose that there also the specific affinity of the cancer cells to the cells of different organs is the only reason for the specific localization of metastases.

The fact, then, that carcinoma forms metastases in the lymphatic glands is not due to the difference in the channels of transportation in the carcinoma and the sarcoma, but to the specific affinity between carcinoma cells and the cells of lymphatic glands. In the case related above, where sarcoma cells were found filling the blood-vessels immediately adjoining a lymphatic gland, no metastasis formed in the organ. The sarcoma cells had easy access to the gland, but apparently could not find there a favorable medium for their proliferation. The relation of this affinity to various factors in the genesis of malignant tumors and the changes in this relationship which may be noted when the parenchymatous organs are abnormal, are subjects of further research by the writers.

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EXPLANATION OF PLATES.

Plate 18.

Fig 1. A metastatic growth in the lung of the Flexner-Jobling carcinoma of the rat formed after an intravenous injection of the tumor emulsion. In the centre of the figure is seen a group of carcinoma cells.

Fig. 2. A metastatic growth of mouse sarcoma in the liver of a rat. The upper margin of the specimen shows a mass of growing sarcoma cells.

PLATE 19.

FIG. 3. Numerous minute blood-vessels in fat tissue, filled with sarcoma cells, in the vicinity of a lymphatic gland. A part of the lymphatic gland is seen to the left of the figure, the blood-vessels to the right.

FIG. 4. Numerous minute blood-vessels in fat tissue in the vicinity of a lymphatic gland. The figure presents a morphological picture identical with the one shown in figure 3, but without the tumor embolism in the blood-vessels.

FIG. 1.

FIG. 2.

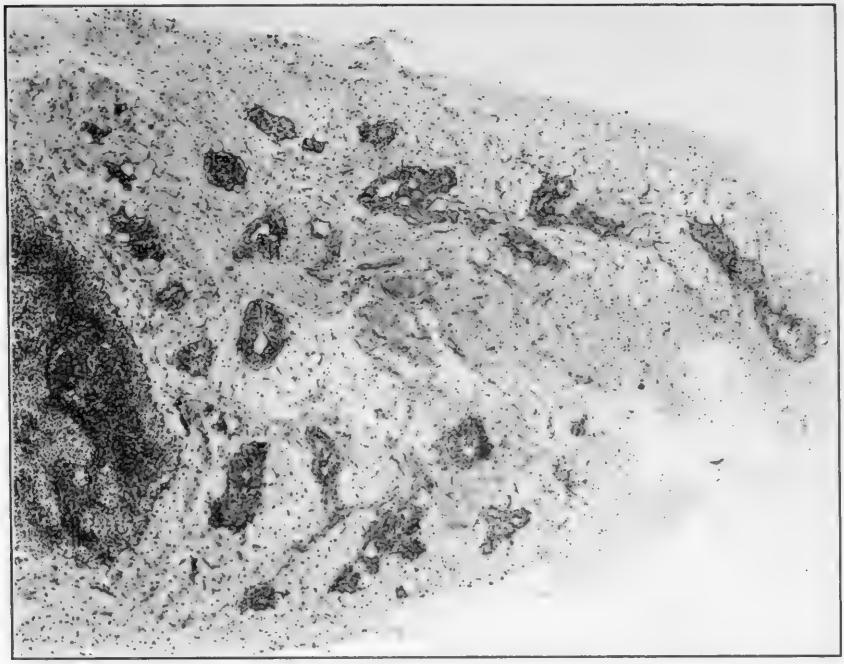

FIG. 3.

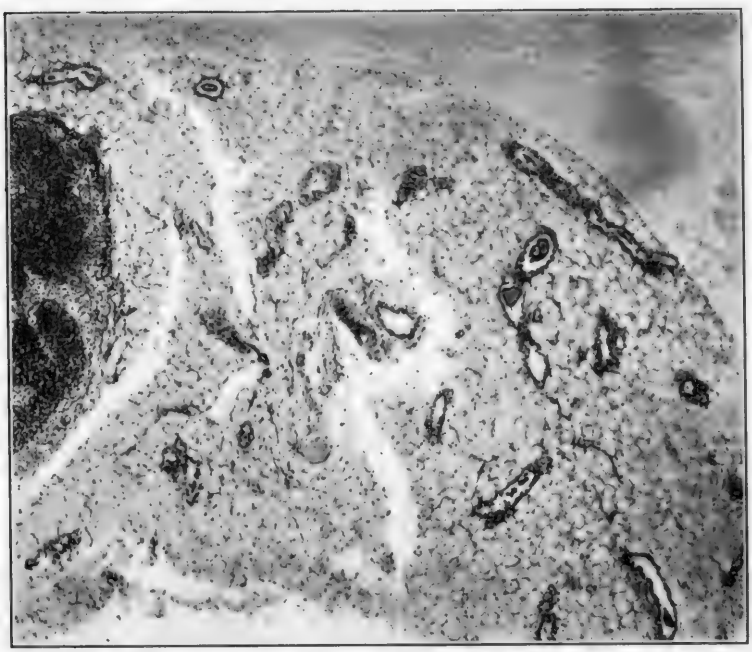

FIG. 4.

EXPERIMENTAL OBSTRUCTIVE JAUNDICE.*

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The disturbances in the body following obstructive jaundice have been extensively investigated, and an abundant literature has resulted. Attention, however, has been directed mainly to the circulatory and digestive features. It is not the aim of this paper to present the facts which have been established regarding the toxic effects of bile upon the circulation and digestion. The former have been previously presented by one of us (1), and the latter are concisely given in von Noorden's Metabolism and Practical Medicine (2).

The purpose of the experiments reported here was to ascertain what disturbance of the calcium metabolism would result from occlusion of the common bile duct in dogs. Upon this more limited aspect of the subject, there is practically no literature with which we are familiar, as no quantitative studies of the calcium balance in obstructive jaundice seem to have been made. In order to facilitate the interpretation of the experiments reported here, it is, perhaps, not amiss to rehearse briefly the main facts regarding the physiology of calcium (3).

Calcium occurs in both animal and vegetable foods, partly in loose combination with albumins of an acid character, but to a much greater extent in the salts of phosphoric and carbonic acids. The calcium content of vegetable foods is much greater than that of animal foods. Those foods which are partaken of by the growing organism and those required for the formation of bone are particularly rich in calcium.

The greater part of the calcium taken into the body passes into the feces, only a small proportion being eliminated by the kidneys. The insoluble inorganic calcium salts are partially dissolved in the stomach, the extent depending upon the amount of hydrochloric acid present in that organ. The stomach, however, absorbs these salts only in the slightest traces. The main organ for their absorp-

* Received for publication, May 22, 1911.

tion is the small intestine. In this the dissolved calcium salts are changed into neutral or acid carbonates and phosphates and into salts of fatty acid. The amount of absorption that takes place depends upon the reaction of the intestines, the amount of carbonic and phosphoric acid present, and upon the various organic acids of fermentation.

After being absorbed into the body, calcium is used to build up tissues, particularly bone. Bone has the highest calcium content and blood plasma the next highest. Calcium is by far the most abundant metallic element in the body and is found in all of its cells and fluids.

Calcium is excreted mainly by the intestine, which Magnus Levy calls the organ of excretion of lime. The kidneys are concerned only in a secondary way. Friedrich Müller observed that the secretion of the intestines hardly contains enough calcium to account for that found in the feces, so that it is probably excreted by the epithelial cells of the villi.

The kidney of a normal adult can eliminate daily only comparatively small amounts of calcium, about 0.1 to 0.5 gram, or 18 to 43 per cent. of the total, while the feces contain 60 to 80 per cent. With increased acidity of the urine, its capacity to dissolve calcium is increased; this explains the greater urinary content of calcium in carnivora. In general, acids increase the quantity of calcium eliminated by the urine, while alkalis decrease it. This is well illustrated by the high calcium content of the urine of diabetic acidosis. When, however, a patient in the latter condition is treated with alkali, the calcium content of the urine diminishes.

As a rule, calcium salts injected subcutaneously and intraperitoneally are indifferently well absorbed and the results yielded by these methods are less conclusive than those given by the intravenous method. However, Rey (4) and others have shown that after either subcutaneous or intravenous injection of calcium salts, the calcium content of the large intestine is increased. Rey has thus localized an important place of elimination.

The kidneys take a share in excretion, as is shown by Mendel and Benedict's (5) experiments, in which calcium chlorid solution was introduced intravenously into animals and the excess found to be eliminated in part by the kidney. In rabbits the amount was 50 to 60 per cent., and in dogs 15 to 20 per cent.

The disturbances in calcium metabolism in disease are interesting. However, it does not seem desirable in this place to discuss them, as the subject has been briefly reported by one of us (6). Suffice it to say here that but little is known concerning the rôle of calcium in the pathological process which it is our purpose to consider.

Experiments previously reported (7) have brought out the fact that calcium has the power of neutralizing the toxic effects of bile pigments on the circulation. It was then shown that if calcium was united to bile pigments, and the resultant compound injected into the circulation, no untoward effects resulted; while if the bile pigments alone were injected the death of the animal speedily occurred with the same toxic manifestations as when whole bile was injected.

Such a protective action upon the part of calcium suggested that perhaps this base was called upon in the body to neutralize the bile and protect the organism in cases of obstruction where bile was being absorbed into the circulation over long periods of time. Some such protective mechanism would make more intelligible those cases of obstructive jaundice in which, with long continued absorption, very few toxic symptoms are apparent.

Furthermore, the disturbance in the coagulation of the blood which so frequently accompanies obstructive jaundice, and the clinical improvement that may follow administration of calcium salts also suggest that calcium, which is essential for the clotting of blood, may be deviated from its normal rôle in this process and used to neutralize the toxic pigments, thus combating a serious danger to the body.

Such considerations led us to make a few preliminary quantitative analyses for calcium in organs from dogs with obstructive jaundice, and, in general, a loss of calcium was found. It seemed desirable, therefore, to make a more complete investigation of the calcium balance in obstructive jaundice produced experimentally in dogs, and the results are reported in this paper.

METHOD.

For each experiment two healthy, active female dogs as near the same weight as possible were taken, one for the production of an obstructive jaundice, and the other for control. They were placed on a known diet and their total calcium metabolism was followed for a week. In one an obstructive jaundice was then produced by tying the common duct in two places and cutting between the ligatures. After the animal recovered from the immediate effects of the operation, the study was continued. All of the animals operated upon developed definite jaundice. When the calcium metabolism had been followed for a week,[1] during which well marked jaundice was present, the jaundiced animal and the control were killed and their organs

[1] The analyses were continued over two to three weeks after the production of jaundice and the figures quoted in the experiment were taken on seven consecutive days at the same period in the jaundiced dog and in the control. The analyses were done in duplicates.

were studied for their calcium content. The animals were bled to death under ether, and then, in order to remove the serum from the organs, both dogs were perfused intravenously with 0.8 per cent. sodium chlorid under the same pressure and with approximately the same amounts.

The organs were dried, weighed, and incinerated, and the calcium was determined by precipitation as calcium oxalate, the titration being made with potassium permanganate. The same technique was employed for the analysis of the diet, urine, and feces.

Experiment I.—Control dog A, weight 13¼ lbs.; jaundiced dog B, weight 14 lbs.

The daily diet in each case was 200 grams of lean meat and 250 c.c. of water given by stomach tube. The animals readily ate the entire amount of meat. Whenever the entire amount was not consumed, the portion refused was weighed and deducted from the intake. A sample of the meat was analyzed each time for the calcium content.

The analysis on the intake and output of dogs A and B for the week previous to the production of jaundice shows that neither of them was in exact calcium equilibrium, but that in both the calcium output slightly exceeded the intake; in dog A by 0.1485 grams, and

EXPERIMENT I.

Control Dog A. Weight 13 1/4 lbs. First Period.

Day.	Food.	Calcium intake in gm.	Urine in c.c.	Calcium in urine in gm.	Calcium in feces in gm.
1	200 gm. meat, 250 c.c. water.	0.0080	280	0.0060	0.0207
2	200 gm. meat, 250 c.c. water.	0.0080	285	0.0122	0.0269
3	200 gm. meat, 250 c.c. water.	0.0080	275	0.0148	0
4	200 gm. meat, 250 c.c. water.	0.0080	240	0.0031	0.0423
5	200 gm. meat, 250 c.c. water.	0.0330	360	0.0050	0
6	200 gm. meat, 250 c.c. water.	0.0330	310	0.0042	0.1380
7	200 gm. meat, 250 c.c. water.	0.0330	210	0.0063	0
Total	1,400 gm. meat plus 1,750 c.c. water.	0.1310	1,960	0.0516	0.2279

Total output calcium = 0.2795 gm.
Total intake of calcium = 0.1310 gm.
Excess output of calcium over intake = 0.1485 gm.

EXPERIMENT I.

Dog B. Weight 14 lbs. First Period.

Day.	Food.	Calcium intake in gm.	Urine in c.c.	Calcium in urine.	Calcium in feces.
1	200 gm. meat, 250 c.c. water.	0.0096	300	0.0080	0
2	200 gm. meat, 250 c.c. water.	0.0096	310	0.0078	0.0950
3	200 gm. meat, 250 c.c. water.	0.0096	355	0.0071	0
4	200 gm. meat, 250 c.c. water.	0.0130	365	0.0054	0
5	200 gm. meat, 250 c.c. water.	0.0130	210	0.0042	0
6	200 gm. meat, 250 c.c. water.	0.0130	250	0.0037	0.0495
7	200 gm. meat, 250 c.c. water.	0.0130	300	0.0045	0
Total	1,400 gm. meat plus 1,750 c.c. water.	0.0808	2,090	0.0407	0.1445

Total output of calcium = 0.1852 gm.
Total intake of calcium = 0.0808 gm.
Excess output of calcium over intake = 0.1044 gm.

in dog B by 0.1044 grams. At this point dog B was operated upon and an obstructive jaundice produced as described above. The analyses of the control and jaundiced dogs are given in experiment I.

The output of calcium in the urine of the control dog was approximately the same, before and after the beginning of the experiment.

Calcium in urine for 7 days before = 0.0516 gm.
Calcium in urine for 7 days after = 0.0436 gm.

In the feces the output in the first and second periods again corresponded very closely.

Calcium in feces for 7 days before = 0.2279 gm.
Calcium in feces for 7 days after = 0.2796 gm.

The balance of calcium output over intake was also close.

Calcium output over intake for 7 days before = 0.1405 gm.
Calcium output over intake for 7 days after = 0.1472 gm.

In the jaundiced dog there was, however, a marked disturbance in the calcium balance.

EXPERIMENT I.

Control Dog A. Second Period.

Day.	Food.	Calcium intake in gm.	Urine in c.c.	Calcium in urine in gm.	Calcium in feces in gm.
1	200 gm. meat, 250 c.c. water.	0.0340	250	0.0037	0.1218
2	200 gm. meat, 250 c.c. water.	0.0340	360	0.0054	0.0603
3	200 gm. meat, 250 c.c. water.	0.0340	350	0.0094	0
4	200 gm. meat, 250 c.c. water.	0.0340	400	0.0054	0
5	200 gm. meat, 250 c.c. water.	0.0340	400	0.0054	0.0510
6	200 gm. meat, 250 c.c. water.	0.0030	225	0.0067	0.0465
7	200 gm. meat, 250 c.c. water.	0.0030	425	0.0076	0
Total	1,400 gm. meat plus 1,750 c.c. water.	0.1760	2,410	0.0436	0.2796

Total output of calcium = 0.3232 gm.
Total intake of calcium = 0.1760 gm.
Excess output of calcium over intake = 0.1472 gm.

EXPERIMENT I.

Jaundiced Dog B. Second Period.

Day.	Food.	Calcium intake in gm.	Urine in c.c.	Calcium in urine in gm.	Calcium in feces in gm.
1	200 gm. meat, 250 c.c. water.	0.0087	325	0.0078	0.0424
2	200 gm. meat, 250 c.c. water.	0.0087	250	0.0045	0.0186
3	200 gm. meat, 250 c.c. water.	0.0130	300	0.0036	0.0771
4	200 gm. meat, 250 c.c. water.	0.0130	225	0.0089	0.0672
5	200 gm. meat, 250 c.c. water.	0.0130	270	0.0089	0.1275
6	200 gm. meat, 250 c.c. water.	0.0130	325	0.0026	0.1700
7	200 gm. meat, 250 c.c. water.	0.0030	290	0.0033	0.0217
Total	1,400 gm. meat plus 1,750 c.c. water.	0.0724	1,985	0.0396	0.5245

Total output of calcium = 0.5641 gm.
Total intake of calcium = 0.0724 gm.
Excess output of calcium over intake = 0.4917 gm.

There was practically little change in output of calcium by urine after the production of the obstructive jaundice.

Calcium in urine for 7 days before = 0.0407 gm.
Calcium in urine for 7 days after = 0.0396 gm.

In the feces, however, was a marked change.

Calcium in feces for 7 days before = 0.1445 gm.
Calcium in feces for 7 days after = 0.5245 gm.

The study of the output over the intake of calcium for this period showed a striking loss of calcium by the jaundiced animal.

Calcium output over intake for 7 days before = 0.1044 gm.
Calcium output over intake for 7 days after = 0.4917 gm.

The analyses of the various organs showed an interesting disturbance in their calcium content.

EXPERIMENT I.

Analysis of Organs.

Per 1,000 parts.	Control dog. Calcium in gm.	Jaundiced dog. Calcium in gm.	Change of calcium in jaundiced dog, in gm.
Blood	0.0700	0.1100	0.0400 +
Heart	0.0938	0.0614	0.0324 −
Muscle	0.0200	0.0335	0.0135 +
Liver	0.0350	0.0760	0.0410 +
Bone (femur)	202.9493	175.3186	27.6307 −
Pancreas	0.1425	0.1400	0.0025 −
Brain	0.0950	0.1056	0.0106 +
Kidneys	0.1470	0.1818	0.0348 +

Except for some slight differences, the analysis here given agrees with the preliminary one published at the end of a previous paper (1). The present analyses, however, represent a longer study under more carefully controlled conditions.

In the jaundiced animal there was an increase in the calcium of the blood, which supports the idea that the circulating pigments may be neutralized by this base. There was, however, a loss of calcium in the heart. This is of interest in relation to bradycardia, and will be discussed later under that heading. The bone of the jaundiced dog showed a striking loss of calcium and the findings point to this organ as furnishing most of the calcium to combat the intoxication.

The kidneys of the jaundiced dog showed a slight gain in calcium, but the other organs showed relatively unimportant changes.

EXPERIMENT II.

Control Dog A. Weight 13 1/4 lbs. First Period.

Day.	Food.	Calcium intake in gm.	Urine in c.c.	Calcium in urine in gm.	Calcium in feces in gm.
1	200 gm. meat, 250 c.c. water.	0.0046	250	0.0050	0.0460
2	200 gm. meat, 250 c.c. water.	0.0046	350	0.0035	0
3	200 gm. meat, 250 c.c. water.	0.0046	190	0.0085	0
4	200 gm. meat, 250 c.c. water.	0.0100	275	0.0130	0.0463
5	200 gm. meat, 250 c.c. water.	0.0100	210	0.0052	0.0535
6	200 gm. meat, 250 c.c. water.	0.0100	250	0.0020	0
7	200 gm. meat, 250 c.c. water.	0.0100	265	0.0106	0
Total	1,400 gm. meat plus 1,750 c.c. water.	0.0538	1,790	0.0478	0.1458

Total output of calcium = 0.1936 gm.
Total intake of calcium = 0.0538 gm.
Excess output of calcium over intake = 0.1398 gm.

EXPERIMENT II.

Dog C. Weight 13 1/4 lbs. First Period.

Day.	Food.	Calcium intake in gm.	Urine in c.c.	Calcium in urine in gm.	Calcium in feces in gm.
1	200 gm. meat, 250 c.c. water.	0.0046	370	0.0054	0
2	200 gm. meat, 250 c.c. water.	0.0046	350	0.0064	0
3	200 gm. meat, 250 c.c. water.	0.0040	350	0.0035	0
4	200 gm. meat, 250 c.c. water.	0.0100	240	0.0048	0
5	200 gm. meat, 250 c.c. water.	0.0100	300	0.0037	0.1980
6	200 gm. meat, 250 c.c. water.	0.0100	310	0.0020	0
7	200 gm. meat, 250 c.c. water.	0.0100	200	0.0038	0
Total	1,400 gm. meat plus 1,750 c.c. water.	0.0532	2,120	0.0296	0.1980

Total output of calcium = 0.2276 gm.
Total intake of calcium = 0.0532 gm.
Excess output of calcium over intake = 0.1744 gm.

A second experiment was carried out under exactly the same conditions as outlined in the above study. The results are given in experiment II.

EXPERIMENT II.

Control Dog A. Second Period.

Day.	Food.	Calcium intake in gm.	Urine in c.c.	Calcium in urine in gm.	Calcium in feces in gm.
1	200 gm. meat, 250 c.c. water.	0.0066	280	0.0028	0
2	200 gm. meat, 250 c.c. water.	0.0066	325	0.0176	0.0765
3	200 gm. meat, 250 c.c. water.	0.0100	375	0.0053	0
4	200 gm. meat, 250 c.c. water.	0.0100	265	0.0055	0
5	200 gm. meat, 250 c.c. water.	0.0100	270	0.0074	0.0700
6	200 gm. meat, 250 c.c. water.	0.0100	250	0.0038	0.0607
7	200 gm. meat, 250 c.c. water.	0.0340	360	0.0046	0.0304
Total	1,400 gm. meat plus 1,750 c.c. water.	0.0872	2,125	0.0470	0.2376

Total output of calcium = 0.2846 gm.
Total intake of calcium = 0.0872 gm.
Excess output of calcium over intake = 0.1974 gm.

EXPERIMENT II.

Jaundiced Dog C. Second Period.

Day.	Food.	Intake of calcium in gm.	Urine in c.c.	Calcium in urine in gm.	Calcium in feces in gm.
1	200 gm. meat, 250 c.c. water.	0.0066	325	0.0033	0
2	200 gm. meat, 250 c.c. water.	0.0066	285	0.0043	0.2172
3	200 gm. meat, 250 c.c. water.	0.0100	285	0.0043	0.0415
4	200 gm. meat, 250 c.c. water.	0.0100	330	0.0066	0.0465
5	200 gm. meat, 250 c.c. water.	0.0100	240	0.0048	0.0560
6	200 gm. meat, 250 c.c. water.	0.0100	410	0.0061	0.0744
7	200 gm. meat, 250 c.c. water.	0.0340	375	0.0045	0.0792
Total	1,400 gm. meat plus 1,750 c.c. water.	0.0872	2,250	0.0339	0.5148

Total output of calcium = 0.5487 gm.
Total intake of calcium = 0.0872 gm.
Excess output of calcium over intake = 0.4615 gm.

Here, again, the dogs were not in exact calcium equilibrium before the obstructive jaundice was produced. There was an excess of output over intake in both cases, amounting in dog A to 0.1398 gram, and in dog C to 0.1744 gram. The obstructive jaundice was produced in the same manner as in the preceding experiments and the analyses follow.

The output of calcium in urine of control dog A for seven days previous to the operation and for seven days after was very close.

Calcium in urine for 7 days before = 0.0478 gm.
Calcium in urine for 7 days after = 0.0470 gm.

In the feces the output was found to be:

Calcium in feces for 7 days before = 0.1458 gm.
Calcium in feces for 7 days after = 0.2376 gm.

The output over the intake was reasonably close.

Calcium output over intake for 7 days before = 0.1398 gm.
Calcium output over intake for 7 days after = 0.1974 gm.

In the jaundiced dog there was, however, a marked disturbance in the calcium balance.

In the urine there was practically no change.

Calcium in urine for 7 days before = 0.0296 gm.
Calcium in urine for 7 days after = 0.0339 gm.

In the feces, however, there was a marked increase in output of calcium after the production of obstructive jaundice.

Calcium in feces for 7 days before = 0.1980 gm.
Calcium in feces for 7 days after = 0.5148 gm.

The calcium balance, as given by comparing intake and output, shows a striking disturbance.

Excess of output of calcium over intake for 7 days before = 0.1744 gm.
Excess of output of calcium over intake for 7 days after = 0.4615 gm.

Here, as in the previous experiment, a marked loss of calcium by the jaundiced animal was found.

The organs were analyzed, as before, dog A being the control for dogs B and C.

EXPERIMENT II.

Analysis of Organs.

Per 1,000 parts.	Control dog. Calcium in gm.	Jaundiced dog. Calcium in gm.	Changes in calcium in jaundiced dog, in gm.
Blood	0.0700	0.1020	0.0320 +
Heart	0.0937	0.0298	0.0679 −
Muscle	0.0200	0.0300	0.0100 +
Liver	0.0350	0.0240	0.0110 −
Bone (femur)	202.9492	191.2796	11.6696 −
Pancreas	0.1425	0.1560	0.0135 +
Brain	0.0950	0.0990	0.0040 −
Kidneys	0.1470	0.1918	0.0448 +

The table shows in the jaundiced animal a gain of calcium by the blood and kidneys, and a loss by the heart and bone. The other organs show relatively slight changes. The findings support those of the first series, and permit of the same deductions.

A third experiment was performed in exactly the same manner as the two previous ones. However, larger dogs had to be taken, and consequently they do not approach quite as close to equilibrium as the other two cases.

EXPERIMENT III.

Control Dog L. Weight 23 lbs. First Period.

Day.	Food.	Intake of calcium in gm.	Urine in c.c.	Calcium in urine in gm.	Feces in gm.	Calcium in feces in gm.
1	250 gm. meat, 250 c.c. water.	0.0340	410	0.0201	0	0
2	250 gm. meat, 250 c.c. water.	0.0340	350	0.0140	8.00	0.1800
3	250 gm. meat, 250 c.c. water.	0.0420	175	0.0088	0	0
4	250 gm. meat, 250 c.c. water.	0.0420	190	0.0135	6.409	0.1362
5	250 gm. meat, 250 c.c. water.	0.0300	160	0.0128	0	0
6	250 gm. meat, 250 c.c. water.	0.0300	360	0.0090	0	0
7	250 gm. meat, 250 c.c. water.	0.0300	310	0.0138	7.500	0.3169
Total	1,750 gm. meat, plus 1,750 c.c. water.	0.2420	1,955	0.0920	21.909	0.6331

Total output of calcium = 0.7251 gm.
Total intake of calcium = 0.2420 gm.
Excess output of calcium over intake = 0.4831 gm.

EXPERIMENT III.

Dog M. Weight 24 lbs. First Period.

Day.	Food.	Intake of calcium in gm.	Urine in c.c.	Calcium in urine in gm.	Feces in gm.	Calcium in feces in gm.
1	250 gm. meat, 250 c.c. water.	0.0340	210	0.0116	10.590	0.1774
2	250 gm. meat, 250 c.c. water.	0.0340	288	0.0125	9.000	0.0338
3	250 gm. meat, 250 c.c. water.	0.0420	105	0.0099	0	0
4	250 gm. meat, 250 c.c. water.	0.0420	148	0.0060	13.500	0.1586
5	250 gm. meat, 250 c.c. water.	0.0300	240	0.0096	14.870	0.1487
6	250 gm. meat, 250 c.c. water.	0.0300	310	0.0105	0	0
7	250 gm. meat, 250 c.c. water.	0.0300	185	0.0068	7.100	0.1659
Total	1,750 gm. meat plus 1,750 c.c. water.	0.2420	1,486	0.0669	55.060	0.6844

Total output of calcium = 0.7513 gm.
Total intake of calcium = 0.2420 gm.
Excess output of calcium over intake = 0.5093 gm.

Here again the dogs were not in exact calcium equilibrium. There was an excess of output of calcium over intake in both cases, amounting in dog L to 0.4831 gram and in dog M to 0.5093 gram. In dog M an obstructive jaundice was produced as in the previous cases. The analyses follow:

The output of calcium in urine of control dog L for seven days previous to the operation and for seven days after was:

Calcium in urine for 7 days before = 0.0920 gm.
Calcium in urine for 7 days after = 0.0712 gm.

In the feces the output was found to be:

Calcium in feces for 7 days before = 0.6331 gm.
Calcium in feces for 7 days after = 0.6694 gm.

The excess of output over intake was close.

Calcium output over intake for 7 days before = 0.4831 gm.
Calcium output over intake for 7 days after = 0.4354 gm.

EXPERIMENT III.

Control Dog L. Second Period.

Day.	Food.	Calcium intake in gm.	Urine in gm.	Calcium in urine in gm.	Feces.	Calcium in feces in gm.
1	250 gm. meat, 250 c.c. water.	0.0860	230	0.0115	0	0
2	250 gm. meat, 250 c.c. water.	0.0860	290	0.0116	0	0
3	250 gm. meat, 250 c.c. water.	0.0216	170	0.0119	0	0
4	250 gm. meat, 250 c.c. water.	0.0216	220	0.0119	0	0
5	250 gm. meat, 250 c.c. water.	0.0300	145	0.0058	0	0
6	250 gm. meat, 250 c.c. water.	0.0300	190	0.0076	0	0
7	250 gm. meat, 250 c.c. water.	0.0300	145	0.0109	17.500	0.6694
Total	1,750 gm. meat plus 1,750 c.c. water.	0.3052	1,390	0.0712	17.500	0.6694

Total output of calcium = 0.7406 gm.
Total intake of calcium = 0.3052 gm.
Excess of output of calcium over intake = 0.4354 gm.

EXPERIMENT III.

Jaundiced Dog M. Second Period.

Day.	Food.	Calcium intake in gm.	Urine in c.c.	Calcium in urine in gm.	Feces in gm.	Calcium in feces in gm.
1	250 gm. meat, 250 c.c. water.	0.0860	290	0.0189	25.000	0.1625
2	250 gm. meat, 250 c.c. water.	0.0860	164	0.0139	16.000	0.1120
3	250 gm. meat, 250 c.c. water.	0.0216	190	0.0162	0	0
4	250 gm. meat, 250 c.c. water.	0.0216	225	0.0333	26.250	0.4725
5	250 gm. meat, 250 c.c. water.	0.0300	158	0.0027	40.800	0.2040
6	250 gm. meat, 250 c.c. water.	0.0300	175	0.0053	43.609	0.3925
7	250 gm. meat, 250 c.c. water.	0.0300	190	0.0086	4.600	0.0322
Total	1,750 gm. meat plus 1,750 c.c. water.	0.3052	1,392	0.0989	156.259	1.3757

Total output of calcium = 1.4746 gm.
Total intake of calcium = 0.3052 gm.
Excess of output of calcium over intake = 1.0694 gm.

In the jaundiced dog there was, however, a marked disturbance in the calcium balance.

There was a slight change in the urine.

Calcium in urine for 7 days before = 0.0669 gm.
Calcium in urine for 7 days after = 0.0989 gm.

And in the feces there was a marked change in the output of calcium after the production of obstructive jaundice.

Calcium in feces for 7 days before = 0.6844 gm.
Calcium in feces for 7 days after = 1.3757 gm.

The calcium balance as given by comparing the intake with the output showed a striking disturbance.

Excess of output over intake of calcium for 7 days before = 0.5093 gm.
Excess of output over intake of calcium for 7 days after = 1.0694 gm.

This experiment also demonstrated a marked loss of calcium, following the development of an obstructive jaundice. In this experiment an exact record was kept of the quantity of feces passed. It is seen that the bulk increases after the production of jaundice, and, furthermore, there is an increase in the fats as shown by the macroscopic appearance of the stools.

No study of the relation of the fats, split and unsplit, in these stools was undertaken. The presence of so much fat in the stools suggests that a part of the increase in calcium in the feces may be due to the chemical fixation of calcium by the fats to form soaps. However, the increase in the calcium content of the blood and the loss of calcium by the bone, together with the fact that the output of calcium by the feces greatly exceeds the total calcium intake, leave no doubt that the most important part of the increased output of calcium is supplied by the body tissues. The calcium loss, therefore, is not to be explained to any great extent by diminished absorption of this base, due to its combination with the fats in the feces.

A study of the calcium content of the organs of the control and jaundiced dogs shows the same interesting changes that were pointed out in experiments I and II, and substantiates the results obtained in those studies.

EXPERIMENT III.

Analysis of Organs.

Per 1,000 parts.	Control dog. Calcium in gm.	Jaundiced dog. Calcium in gm.	Change of calcium in jaundiced dog, in gm.
Blood	0.0450	0.0600	0.0150 +
Heart	0.0647	0.0349	0.0298 −
Liver	0.0720	0.1180	0.0460 +
Bone (femur)	137.1532	102.3217	34.8315 −
Kidney	0.18078	0.25665	0.07587 +

It is quite striking that, although in every case the animal in which the jaundice was produced showed abundant pigment in the urine and absence of it in the stools, nevertheless, there was practically no increase of calcium in the urine, and the elimination of this base by the feces was exactly like that in the normal animal.

The increase of the calcium in the blood and kidneys after jaundice would indicate that the calcium is carried to the kidneys in combination with the pigments, but only the pigment escapes into the urine together with approximately the amount of calcium that the kidney normally excretes.

What becomes of the excess brought to the kidneys? The answer is suggested by the findings in the analysis of the feces. Here the amount of calcium is increased after the production of jaundice, and, as the large intestine is, according to Rey, the main path of excretion of calcium, such findings would indicate that the excess of calcium is eliminated by the feces through the large intestine.

The mechanism of excretion of calcium pigment salt in obstructive jaundice would then appear to be as follows: the calcium pigment salt is carried to the kidney, the pigment is passed into the urine in large quantities, but the calcium in quantities which are only slightly, if at all, increased. The excess of calcium is liberated by way of the intestine.

It seemed to us that there was an experimental way of testing this hypothesis; namely, by injecting into the circulation a bile pigment salt containing a known percentage of calcium, and following the path of elimination of the calcium. Such an experiment was undertaken. A calcium pigment salt was prepared by precipitating

filtered pig bile with calcium lactate, washing it with water and ether and carefully drying it. This precipitate was then analyzed for calcium, dissolved in 95 per cent. alcohol, diluted to a known volume with 0.8 per cent. salt solution, and injected into the jugular vein of the dog. The calcium balance of the dog was followed before and after the injection of the salt.

A female fox terrier weighing twenty-five pounds was put on a meat diet which was analyzed for its calcium content. The output of calcium in the urine and feces was followed for a week before the injection of calcium pigment salt.

EXPERIMENT IV.

Before Injection of Calcium Pigment Salt.

Day.	Food.	Intake of calcium in gm.	Urine in c.c.	Calcium in urine in gm.	Feces in gm.	Calcium in feces in gm.
1	250 gm. meat, 250 c.c. water.	0.0460	245	0.0086	0	0
2	250 gm. meat, 250 c.c. water.	0.0460	105	0.0103	12.000	0.1417
3	250 gm. meat, 250 c.c. water.	0.0440	300	0.0105	0	0
4	250 gm. meat, 250 c.c. water.	0.0440	210	0.0105	11.300	0.4209
5	250 gm. meat, 250 c.c. water.	0.0620	240	0.0120	0	0
6	250 gm. meat, 250 c.c. water.	0.0620	150	0.0108	0	0
7	250 gm. meat, 250 c.c. water.	0.0620	260	0.0108	0	0
Total	1,750 gm. meat plus 1,750 c.c. water.	0.3660	1,510	0.0735	23.300	0.5626

Total output of calcium = 0.6361 gm.
Total intake of calcium = 0.3660 gm.
Total output of calcium over intake = 0.2701 gm.

Operation.—Under ether anesthesia, 2.8 grams of calcium pigment salt in 35 c.c. alcohol (95 per cent.), diluted to 100 c.c. with 0.8 per cent. salt solution were injected, at the rate of 4 c.c. per minute, into right external jugular vein. The amount of calcium in the pigment salt was 0.3542 gm. The analyses were continued as above for one week.

EXPERIMENT IV.

After Injection of Calcium Pigment Salt.

Day.	Food.	Calcium intake in gm.	Urine in c.c.	Calcium in urine in gm.	Feces in gm.	Calcium in feces in gm.
1	250 gm. meat, 250 c.c. water.	0.0480	415	0.0332	0	0
2	250 gm. meat, 250 c.c. water.	0.0480	300	0.0195	0.770	0.0035
3	250 gm. meat, 250 c.c. water.	0.0580	215	0.0086	11.100	0.2869
4	250 gm. meat, 250 c.c. water.	0.0580	185	0.0102	0	0
5	250 gm. meat, 250 c.c. water.	0.0600	185	0.0102	0	0
6	250 gm. meat, 250 c.c. water.	0.0600	185	0.0101	0	0
7	250 gm. meat, 250 c.c. water.	0.0600	190	0.0114	15.900	0.4293
Total	1,750 gm. meat, 1,750 c.c. water.	0.3920	1,675	0.1032	27.770	0.7197

Total output of calcium = 0.8229 gm.
Total intake of calcium = 0.3920 gm.
Total excess output of calcium over intake = 0.4309 gm.

A study of the calcium balance is of interest.

	Calcium intake in gm.	Calcium in urine in gm.	Calcium in feces in gm.
Before injection	0.3660	0.0735	0.5626
After injection	0.3920	0.1032	0.7197
	Total calcium intake in gm.	Total calcium output in gm.	Excess calcium output in gm.
Before injection	0.3660	0.6361	0.2701
After injection	0.3920	0.8229	0.4309

Therefore the excess of the output of calcium over the intake was:

After injection, 0.4309 gm.
Before injection, 0.2701 gm.
Difference = 0.1608 gm.

This is due to the excess of calcium injected in the bile pigment compound. The greatest percentage of the increased calcium is put out by the feces, as we found in our previous studies of obstructive jaundice.

The results of this experiment appear to support the assumption that in obstructive jaundice the greater percentage of calcium is excreted by way of the feces.

It may prove of interest to consider some of the main features of obstructive jaundice in view of the findings enumerated in the preceding experiments.

Bradycardia.—The slowing of the heart brought about when bile is injected into the circulation is caused by the increased tone of the vagus nerve (8). When the latter is cut the bradycardia disappears. Atropin (9) also will remove the slowing of the heart produced by the injection of bile. Howell (10) has investigated the effect of stimulation of the vagus nerve, and found that there was always an elimination of potassium. Whether this mechanism is operative in the bradycardia of jaundice is not known, but there is another element suggested by the preceding analyses, which may be of importance in the interpretation of this type of bradycardia. Compared with their controls the hearts of each jaundiced animal showed a loss of calcium. This loss of calcium removes a stimulating force from the heart and might to some extent account for the slowing of the heart rate. A slowing of the heart rate was noted in the jaundiced animals, although no accurate record was kept.

Delayed Blood Coagulation.—A most interesting and perplexing complication of obstructive jaundice is the frequently retarded coagulation of the blood. This is in some cases a marked sign of biliary toxemia. The explanation of its occurrence is difficult, and no convincing facts have yet been brought forth.

Several points must be considered in attempting to explain this phenomenon. For the normal clotting of the blood, fibrinogen, thrombin, and calcium are essential. Howell gives the following scheme as a convenient expression of the mechanism of blood clotting.

Prothrombin
+
Calcium
+
zymoplastic substance } = thrombin

Thrombin plus fibrinogen = clot.

In offering a satisfactory explanation, a disturbance in any of the above factors must be considered.

In some of the analyses in jaundiced dogs by Dr. Whipple, it was found that there was no diminution in the fibrinogen; so this factor can be safely ruled out.

The results of our experiments, as well as several analyses by Dr. Whipple, leave no doubt that calcium is increased in the blood in jaundice. However, this increased calcium is bound to the pigments in an effort to neutralize their toxic effects and so may not be at all, or only slowly, available for the clotting of blood. Experiments are being conducted to test this hypothesis.

No observations upon the thrombin content of the blood in jaundice are known to us. Such determinations are now being made, but are not available for this paper. In a subsequent communication, the results of some studies upon this point will be reported.

CONCLUSIONS.

1. In obstructive jaundice, produced in dogs, there is a loss of calcium by the jaundiced animal.

2. The calcium is given up mainly by the bone, to neutralize the toxic bile pigments circulating in the blood and tissues.

3. This neutralization affords protection to the body, but may lead to secondary disturbances, such as bradycardia and change in the blood coagulation time.

4. These two latter signs of biliary toxemia can, perhaps, in some measure be explained by the disturbance of calcium.

5. The path of elimination of the increased calcium is mainly by the feces, and only to a small extent by the kidneys.

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THE FORMATION OF PRECIPITATES IN THE BLOOD IN VITRO BY ACID SALVARSAN SOLUTIONS.*

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H. E. Hering[1] called attention to the difference in toxicity between the acid and the alkaline solutions of salvarsan. Since that time, numerous writers have commented upon the comparative toxicity of these two solutions, but as far as I have seen, only two—Willige[2] and Auer[3]—have mentioned the concentration of the acid solution in connection with its toxicity. Auer showed recently that the toxicity of this solution varies inversely with the dilution.

In connection with the experiments described in a former paper,[4] a large number of mixtures, *in vitro,* of blood and acid solutions of salvarsan of different concentrations were examined for precipitates. In the course of these experiments, the following facts were noted which may be of importance and which may possibly be connected with Auer's observations on the relation between toxicity and the concentration of the acid solution.

Very weak solutions of acid salvarsan (0.1 per cent.) produce with blood *in vitro* no precipitate, or at least no precipitate that is easily seen. The blood retains its normal consistency. This is true even if the volume of the salvarsan solution be from one to three times that of the blood to which it is added. The amount of precipitate produced in the blood increases with the concentration of the acid salvarsan solution employed, so that after the addition of a 1 per cent., or stronger, solution of salvarsan, the precipitate may be so heavy that the blood practically loses its fluidity and the test tube can sometimes even be inverted without spilling its contents.

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[1] H. E. Hering, *München. med. Wchnschr.*, 1910, lvii, 2621.

[2] Willige, *München. med. Wchnschr.*, 1910, lvii, 2403.

[3] Auer, *Arch. Int. Med.*, 1911, vii, July number.

[4] Joseph, *Jour. Exper. Med.*, 1911, xiv, 99.

The loss of fluidity is due to the formation of a precipitate and not to clotting.

While, as stated before, a comparatively large volume of a 0.1 per cent. solution of acid salvarsan may be added to blood without a precipitate being formed, a very small amount of a more concentrated solution of salvarsan (0.5 to 1 per cent. or over) gives a definite precipitate. For example, in one experiment, equal parts of a 0.1 per cent. solution of acid salvarsan and blood gave no precipitate, while 1 part of a 0.5 per cent. acid solution to 54 parts of blood gave a definite precipitate. The precipitate is formed apparently before the salvarsan can be diluted by the large volume of blood to which it is added.

In brief, then, there is a fairly definite relation between the concentration of the acid salvarsan solution used, and the quantity and consistency of the precipitate which it forms when added to the blood *in vitro*.

In the paper[5] mentioned above, it was shown that a precipitate is formed in the blood stream by acid salvarsan solutions, just as *in vitro;* also, that the precipitate nearly always disappears from the blood stream during its passage through the lungs. Now if the more concentrated solutions of acid salvarsan produce heavier precipitates than more dilute solutions when injected intravenously, one might expect concentrated solutions to be the more dangerous. As a matter of fact, in a number of cases in which death was reported in man and animals, following intravenous injections of acid salvarsan solutions, we find that solutions of high concentration were used. Fraenkel and Grouven[6] reported the death of a patient who received a 2.5 per cent. solution; Willige[7] lost a case after using a 2 per cent. solution; and Miessner[8] reported the death of several cattle that received a 5 per cent. solution.

[5] Joseph, *loc. cit.*
[6] Fraenkel and Grouven, *München. med. Wchnschr.*, 1910, lvii, 1771.
[7] Willige, *loc. cit.*
[8] Miessner, *Deutsch. med. Wchnschr.*, 1911, xxxvii, 491.

EXPERIMENTAL IMMUNITY WITH REFERENCE TO THE BACILLUS OF LEPROSY.

PART I.

A STUDY OF THE FACTORS DETERMINING INFECTION IN ANIMALS.*

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Plates 20 and 21.

INTRODUCTION.

The discovery of microörganisms and their identification as causative agents in the production of specific diseases has led in recent years to the newer science of immunology. It may be stated that our present conception of the defensive and offensive mechanism of the host toward parasitic invasion is the direct outcome of studies upon the biochemical properties of bacteria and protozoa. Furthermore, the cultivation and study of the biological characteristics of microörganisms are of the greatest value in rendering possible the study of immune processes. Immunology has presented in particular three problems for elucidation: (1) the factors determining the infection of man and animals, and the development of changes in the tissues—the result of bacterial and protozoal action; (2) the method through which parasites exert their detrimental influence, whether by toxin or simply by mechanical injury to the tissues; and (3) the factors determining the manner of production of protective and curative substances on the part of the infected animal. Naturally, in such a study we must take into consideration the possibility of protective properties on the part of the invading parasites; that is, their immunity against injurious substances already present or subsequently produced in the body of the

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host. This applies especially to a disease like leprosy in which the specific excitor lives and multiplies in the animal body over an indefinite period, and which, in the light of our present knowledge of parasitism, can be explained only upon the ground that a mutual relation exists between host and invader. To determine in what way this reciprocal relation of parasite and host is established may ultimately lead to a means by which such a mechanism can be disarranged and, in consequence, the disease controlled.

In the extensive experiments and observations during the past twenty years, the greater amount of effort has been expended in the consideration of the manner of the production of immune bodies upon the part of the host, together with quantitative and qualitative methods for the determination of such bodies. Thus specific substances have been recognized, to which the names antitoxin, agglutinin, precipitin, amboceptor, opsonin, etc., have been given. The presence and importance of complement or alexin is also being appreciated; and a more adequate knowledge of the part played in the defensive and offensive powers of the host by the cellular elements has been acquired.

In the present communication, the results of animal experiments which throw light upon the type of the immune bodies induced on the part of the host by the inoculation of leprosy bacilli will not be considered extensively, as the purpose of this paper is to draw attention more especially to certain experiments which seem to indicate the factors determining leprosy infection. The experimental development of different types of host immune bodies and their significance will be discussed in a future publication.

Until quite recently, difficulty has been experienced by investigators in producing experimentally leprous lesions in animals. With the exception of the Japanese dancing mouse, most animals were assumed to possess a natural immunity. One of us (Duval[1]) has been successful in producing, with comparative regularity, lesions and even generalized leprosy in the monkey (*Macacus rhesus*). Couret[2] has likewise been able to produce in many cold-blooded animals multiplication of the leprosy bacillus with reactive lesions

[1] *Jour. Exper. Med.*, 1911, xiii, 374.
[2] *Jour. Exper. Med.*, 1911, xiii, 576.

on the part of the host. The guinea pig, goat, and horse, as well as ordinary white mice, have been found by us to be subject to infection. Clegg[3] also reports the production of lesions in the guinea pig and in the rabbit.

The number of animals, then, regarded as naturally immune is diminishing. It may, therefore, be asked with reason, why up to the present time it has been found impossible to infect animals which more recently have shown themselves susceptible to inoculation. An analysis of the following experiments offers, we think, an explanation, and at the same time helps to explain the phenomena noted in the development of the disease in human subjects; namely, the apparent immunity of most individuals and the fact that, as a rule, only those living in long and intimate contact with leprous patients have become infected.

Before proceeding to a detailed description of the experiments, it is, perhaps, proper to state the impression which has been gained by us as a result of the study of these experiments. Briefly, it appears that two factors are of great importance in determining the successful inoculation of animals, if we leave out of consideration, for the present, the variation in virulence of bacilli from different sources. The cause of the unsuccessful attempts to infect animals seems to be the result of insufficient dosage and the fact that a second injection is necessary to establish the infection. We have found that constant takes occur in animals only when sufficiently large doses of the organism are used. Furthermore, second or third injections are much more potent to induce disseminated lesions and the multiplication of the bacilli in the tissues, than is the first, although occasionally with certain species of animals success follows the initial injection provided the dose is very large. Following second and third inoculations, the lesions appear more rapidly, and there is a greater tendency for the organisms to multiply and metastasize than with primary injections. Again, the lesions resulting from subsequent inoculations grow more readily and persist longer than those following the first injection and may

[3] *Bul. Manila Med. Soc.*, 1910, ii, 308.

be produced by smaller doses of bacilli. The primary injection, which, we assume, sensitizes the animal, may consist of dead or living bacilli; either one serves equally as a sensitizing agent.

EXPERIMENTAL PART.

In the experiments, eight monkeys (*Macacus rhesus*), four goats, two kids, eighteen guinea pigs, four white mice, and one horse were employed. For convenience of description, the monkeys, goats, and guinea pigs will be designated alphabetically. In the inoculations, both dead and living bacilli were used, the devitalizing of the organisms being accomplished by means of heat and by treatment with tricresol. It has been determined that heating of the leprosy culture, washed down in saline solution from the surface of agar slants, at 62° C. for one hour kills the organisms. One hour exposure to five per cent. tricresol has the same effect.

Experiment I.—Two monkeys (A and B) were inoculated subcutaneously with living lepra bacilli in doses, approximately, of 400 million organisms. No multiplication or lesions developed until the third injection, when lesions resulted not only at the site of inoculation but also at distant parts, and the animals presented many of the clinical manifestations of human leprosy.

The lesions which developed, however, disappeared after several months. Two months after the disappearance of all signs of the disease both animals were again inoculated upon the inner surface of the thigh with approximately ten billion dead bacilli which had been killed by heating. Twenty-four hours later both monkeys showed small firm nodules, 1 cm. by 5 cm. in size, about the point of inoculation. The following day, *i. e.* forty-eight hours after injection, the nodule in one monkey was retrogressing, while in the other it had increased in size and was red and tender. This nodule when excised was found to consist of a central area containing a purulent yellowish material which, upon microscopic examination, was found to be composed almost entirely of polymorphonuclear leucocytes and numerous acid-fast bacilli. Many of the bacilli were apparently phagocytosed by the pus cells. Three days after the inoculation the nodule in the monkey not operated upon had disappeared; while the only remains of the nodule in the other animal was a small granulating ulcer.

Experiment II.—On March 8, two monkeys (C and D) were inoculated with about ten billion dead bacilli into the subcutaneous tissue over the chest. No reaction was noted at any time following the inoculation. On March 22, both monkeys were again injected, one with dead, the other with living organisms, approximately ten billion bacilli being used in each case. Twenty-four hours after the second inoculation, both animals had small firm nodules upon the inner surface of the thigh at the site of inoculation. In the animal receiving the living organisms, the lesion was 1.8 cm. by 1.2 cm. in extent; while in the other, the

lesion was somewhat smaller. On the following day, the lesion in the monkey which had received the living bacilli had increased in size and was firmer; the nodule in the monkey which received the dead bacilli was smaller than at the previous observation.

Experiment III.—In this experiment, an effort was made to determine, if possible, the size of the dose necessary to infect monkeys by a single inoculation.

Two monkeys (E and F), were inoculated with approximately forty billion living bacilli into the subcutaneous tissue below the nipple. For the first three days, the animals showed neither local nor constitutional reaction, with the exception of a slight edema, which disappeared by the third day. On the fourth day, in both monkeys, small pulpy protuberant masses, which measured 1.8 by 2 cm. in diameter, could be palpated. These swellings were not red, and apparently not tender. The nodules increased slightly in size during the fifth and sixth days, but at no time were they more than barely visible to the naked eye; on palpation, however, they were readily demonstrable. From this time on, the masses decreased in size until the sixteenth day when they had entirely disappeared.

Three weeks after the original inoculation, these monkeys (E and F) were again inoculated, ten billion bacilli being used. Two days after injection, the nodules in the thigh were very prominent, firm, and apparently somewhat tender, measuring 1.5 cm. in diameter in both animals. The nodules continued to increase slowly for several days and remained firm and distinct and somewhat reddened for two weeks.

This experiment with others proves that, although it is possible to induce the production of lesions with the primary inoculation, a very large dose must be used. Such lesions are, moreover, transient and small, never reaching the size of those easily developed upon secondary inoculation by smaller doses.

In experiment IV, we sought to ascertain whether it might be possible to transfer from the serum of an inoculated animal the sensitizing substance which, from former experiments, seemed to be the determining factor in the production of lesions in animals upon second and subsequent injections.

Experiment IV.—Two monkeys (G and H) were inoculated in precisely the same manner. Ten billion living bacilli were injected into the subcutaneous tissue over the chest of both animals, and at the same time 12 c.c. of immune goat serum were given into the thigh. The serum was procured from a goat (Grey Billy) which had developed leprosy lesions after repeated inoculations, in spite of the fact that *in vitro* his blood had early showed fairly well marked bactericidal properties. At no time did monkey G present the slightest local or constitutional reaction to the bacilli. The other animal (H) showed only a small, firm, pea-sized nodule, which appeared upon the fifth and disappeared by the eighth day.

This attempt to infect the animals with *Bacillus lepræ* must, therefore, be considered unsuccessful, since practically no reaction developed in either monkey, although the dose of bacilli was sufficiently large to induce the production of definite nodules in the sensitized animals which were used as controls.

Experiment V.—Two goats (male (A) and female (B)) were inoculated subcutaneously on December 20 with approximately 500 million dead bacilli (killed with tricresol), and a week later a second injection of double the quantity of dead bacilli was given. Ten days after receiving the initial dose, the animals were injected at weekly intervals over a period of three months with doses commencing at 25 million and increasing to approximately 200 billion.

Each inoculation after the first three gave rise to small nodules 1.5 cm. in diameter, which were neither tender nor red. These nodules gradually increased in size for about two weeks, and then disappeared entirely in five or six weeks after their first appearance was noted. Unfortunately no note was made of the time of onset after inoculation, as our attention had not been attracted to the apparent significance and importance of this interval at that time.

After the inoculation on February 17, in which over 200 billion bacilli were injected, a hemispheral mass appeared upon the neck (the site of inoculation) of goat A, which gradually increased in size until March 18, on which date it had attained a diameter of 5.5 cm. On opening the nodule, it was found to contain a friable but not purulent material, which was mottled greyish red in color. This material contained innumerable acid-fast bacilli. The organisms were for the most part clustered together in enormous masses (globi) within large mononuclear and multinuclear cells; and instead of being small coccoid bacilli, they now presented the long beaded appearance that is characteristic of the organism in the human tissues. A pure growth of *B. leprae* was obtained upon special media from the transplanted grumous material. It is noteworthy that the growth did not appear until six days after incubation at 32° C., and it required several subplantations before the bacilli regained their former rapidity of growth and unbeaded coccoid form. The nodule commenced to recede five weeks after its appearance, and by April 1 had practically subsided, leaving a small granulating ulcer at the site of incision. At no time did any material escape from the opening, so that the decrease in the size of the nodule cannot be considered as due to a discharge of its contents. Notwithstanding the progressive growth of the nodule, inoculations were continued, although in somewhat smaller doses, but no further lesions developed subsequently. Previous to the inoculation of February 17, and constantly since that time, the serum from this animal contained antibodies, but they have never increased in any considerable quantity. There was approximately as much immune body in the serum after the first two or three injections as at any subsequent time.

We wish here to draw attention to the point brought out by this experiment to which we shall have occasion to refer again. In the first place, we find that, in spite of the fact that the serum from the

male goat possessed immune bodies to a fairly high degree, the bacilli were able to grow and to develop characteristic lesions.

Experiment VI.—Two kids were born to goat B, which had received twelve doses of leprosy bacilli. The serum from both these young animals was found to fix complement in the presence of *B. leprae* as antigen, in dilutions and in quantities similar to those of the mother. Specific agglutinins, however, were not demonstrable in the serum, as no clumping of the bacilli occurred even in mixtures allowed to stand for twenty-four hours; whereas the mother's serum tested for specific agglutinins gave a very positive reaction in the dilution of 1 to 50.

The lytic properties noted previously in the mother's serum had disappeared, as was evidenced by the profuse growth of the bacilli, which was obtained from mixtures of the organisms and the undiluted serum kept for twenty-four hours at 37° C.

As we thought that perhaps the kids would have naturally a hypersensitiveness to the bacilli, both were inoculated when three weeks old, one receiving about 15 million living, the other a similar quantity of dead bacilli into the under surface of the thigh. The day following the injection the animals were slightly lame, although no definite thickening could be made out. The lameness disappeared at the end of five days without any appreciable lesions having developed.

Apparently then the hypersensitiveness which follows inoculation of the bacilli is not transmitted to the offspring, unless it be considered that in these animals the immunity developed by the mother was sufficiently great to protect the kids against infection.

Owing to the difficulty experienced in fixing foreign complement by means of amboceptor from the goat, after repeated experiments it has been found more satisfactory and trustworthy to utilize the normal or natural complement present in goat serum, and to use as hemolytic body the natural amboceptor against guinea pig corpuscles. The method used is similar to that described by one of us (Gurd[4]) in carrying out the Wassermann reaction. These two bodies (complement and amboceptor) have been found to be constant in their combined action upon guinea pig corpuscles, being active in quantities similar to those in human serum. By means of this method, it was found that the two immunized goats, as well as the kids, fixed large quantities of complement present in normal goat serum.

The following table gives the protocols of the reactions. It is

[4] *Jour. Infect. Dis.*, 1911, viii, 39.

seen that 0.04 cubic centimeter of the immunized serum of all four goats possessed sufficient antibody to fix the complement present in 0.1 cubic centimeter of goat serum.

TABLE I.

Tested serum.	Antigen+ 1.0 c.c. serum.	Antigen+ 0.05 c.c. serum.	0.05 c.c. serum.	0.035 c.c. serum.	Antigen+ 0.05 c.c. immune serum+0.05 c.c. normal serum.	Antigen+ 0.025 c.c. immune serum+0.075 c.c. normal serum.
Normal goat.....	H	H	H	H		
Normal goat.....	H	H	H	H		
Grey Billy (immune)	N	N	H	H	N	I
Nanny (immune)...	N	N	H	H	N	I
Male kid.....	N	N	I	I	N	N
Female kid.....	N	N	I	I	N	N

H=hemolysis; N=no hemolysis; I=incomplete hemolysis.

The leprosy bacilli used as antigen were killed by heating almost to dryness at 60° C. for forty-eight hours, after having been washed down from the surface of 1.5 per cent. alkaline fish agar in sterile distilled water. The bacilli were then suspended in salt solution so that each cubic centimeter contained about 800 million organisms. This material was standardized so that double the quantity necessary to fix complement with positive sera showed no hemolytic, and but slightly anticomplementary properties. Of the suspension used in the tests reported in this paper, six capillary drops were found to be satisfactory in every way.

Experiment VII.—On February 20, a horse was inoculated with approximately 100 billion living bacilli of *B. leprœ* in the subcutaneous tissues of the neck. Six days after the injection, there developed a firm swelling, somewhat tender, about the site of inoculation, which increased in extent for three or four days until it attained a size of 12 by 10 cm. in diameter. The nodule then commenced to recede and had completely disappeared at the end of two weeks. A second injection was given on March 2, and in twenty-four hours a nodule developed which in size was equal to that resulting from the first injection. Within forty-eight hours the mass fluctuated, and on the third day it broke, discharging a purulent material containing necrotic tissue and innumerable acid-fast bacilli. The organisms were scattered and extracellular.

All attempts to cultivate *Bacillus leprœ* from this material failed, which suggests that the bacilli, although maintaining their normal morphology, had been destroyed in the tissues.

The third injection resulted in a lesion similar to that of the second. The dose given in all three injections consisted of approximately the same number of bacilli. The fourth and fifth injections gave rise, from the start, to proliferative lesions which increased steadily in size for several weeks. Microscopic examination of excised bits of the nodule showed the typical lesion of leprosy with large numbers of multinucleated cells containing enormous masses of viable acid-fast bacilli. This was proven by cultural tests.

The horse serum tested at this time showed no bacteriolytic substance; however, specific agglutinins were present in moderate amount, and there was clumping of the bacilli in the dilution of 1 to 200.

Experiment VIII.—On March 7, six guinea pigs (series A) of approximately the same weight (250 grams) were inoculated subcutaneously in the region of the groin with 800 million dead leprosy bacilli. Another lot of six guinea pigs (series B) were, on the same day, injected intraperitoneally with a similar amount of killed leprosy bacilli. Subsequently these twelve animals showed no evidence of either peritonitis or local reaction at the site of inoculation. Six of the guinea pigs, three of which had received the primary dose intraperitoneally and three subcutaneously, were given a second injection of eight million killed leprosy bacilli on April 7, one month after the first inoculation. Two of the animals developed a well marked inflammatory reaction at the site of the second injection. However, the swelling induced in the subcutis, the result of the second injection, had completely subsided in forty-eight hours after its first appearance. These six guinea pigs were inoculated for a third time on May 9, a suspension of approximately two million living leprosy organisms being used instead of killed bacilli. The viable bacilli were obtained by washing down in normal salt solution a sixty-four hour growth from an alkaline fish agar slant.

On this date, the six guinea pigs which had received but one injection of dead bacilli were inoculated with two million viable leprosy bacilli, the same route being used in each case as in the first injection.

All six guinea pigs injected intraperitoneally, half of which had received but one injection, and half two injections of dead organisms, showed signs of peritonitis within twenty-four hours after the inoculation. In two of the animals signs of acute peritonitis continued for three days. During the second day, the animals were so ill that it was thought they would not recover. However, both animals recovered, and in five days after the injection appeared to be in normal health.

Two other pigs of the series injected intraperitoneally gradually lost weight and died, one on May 14, the other on May 20, two and three weeks after the injection of the living bacilli.

Autopsies showed disseminated leprosy in practically all organs of the body (figure 3). In general, the gross appearance of the lesions simulated closely that in guinea pigs dying of tuberculosis. The most striking difference was the

color of the larger caseated areas, which were distinctly golden yellow. The spleen and liver showed the most extensive involvement. Both organs were greatly enlarged and studded with leprous nodules ranging from 1 mm. to 1 cm. in diameter. The smaller areas were firm and greyish white in appearance, while the larger lesions showed central caseation. The spleen was the seat of large anemic infarcts in addition to multiple leprous tubercles of varying size, which were readily distinguished microscopically as leprous lesions by the characteristic appearance of the large cells containing globi and acid-fast bacilli. Pure cultures of *B. leprae* were recovered on special medium from the lesions in both animals.

The four remaining guinea pigs that were injected intraperitoneally were killed at intervals of two, four, ten, and fourteen days after the injection of living bacilli. All of these animals showed more or less extensive leprosy lesions in the abdominal viscera.

Experiment IX.—Eight full-grown guinea pigs were inoculated on March 7 with living leprosy bacilli, four subcutaneously and four intraperitoneally. The dose was approximately 400 million organisms suspended in 5 c.c. of normal saline solution. Twenty-four hours after the injection, the animals appeared to be in perfect health; even those which had received the dose subcutaneously showed no evidence of swelling or tenderness at the site of inoculation. Two weeks after the first injection, four of the animals (two of which had received the bacilli intraperitoneally and two subcutaneously) were given a second injection of 400 billion viable leprosy bacilli. The other four animals of this series were kept for controls.

The two guinea pigs which received the second injection of living bacilli into the peritoneum showed early signs of peritonitis. Twenty-four hours after the injection, the animals refused food and remained huddled in their cages, showing signs of distress. Four days after the second injection the pigs had apparently recovered from the acute symptoms.

The two guinea pigs which had received the second dose of living bacilli subcutaneously developed a tumefaction at the site of inoculation. The nodule in each instance appeared on the third or fourth day and from this time on gradually increased in size. On the fifth day after the injection, the mass in the subcutis of one guinea pig had attained the size of a hen's egg (figure 2). The overlying skin was purple and on palpation the swelling gave evidence of central fluctuation. This animal was found dead in the cage seven days after the injection.

At autopsy, the subcutaneous nodule was found to contain a thick, yellowish white, caseous material which, on microscopic examination, was found to consist almost entirely of mononucleated cells, many of which were crowded with acid-fast bacilli. A pure culture of *B. lepræ* was obtained from this material on special medium. There was no mixed infection. Aside from a slight enlargement of the inguinal glands on the same side as the nodule, no other lesions existed.

The subcutaneous mass at the site of inoculation in the second guinea pig measured 6 by 2 by 4 cm. in diameter two weeks after the injection. The overlying skin was firmly adherent, of a purplish red color, and greatly thickened. Seventeen days after the inoculation the mass ruptured, discharging a thick,

creamy yellow material. From this day, the animal lost weight progressively, and eventually died of the infection twenty-eight days after the second inoculation.

At autopsy the spleen, liver, and lungs were thickly studded with leprous nodules. The spleen was three times its normal size and showed extensive involvement. Large anemic infarcts together with small, yellowish white nodules practically covered the whole organ. Here, as in the other animals, the gross appearance of the lesions greatly resembled tuberculosis. It may be mentioned in this connection that this orange yellow color of the necrotic material in the experimental lesions is also characteristic of the pus in the subcutaneous leprous abscesses in human cases. The color is due in both instances almost entirely to the chromogenic property of the leprosy bacilli.

The four control guinea pigs were killed at varying intervals, and at autopsy were found to be negative with respect to leprous lesions.

These experiments with guinea pigs tend to show that animals which were previously thought to be insusceptible to leprosy are readily infected provided the animal receives a sensitizing dose of either living or dead bacilli. They also prove that the first dose must be large and that an interval of two weeks at least should elapse before giving the second injection. The second or infecting dose may be relatively small as compared to the initial sensitizing dose. Furthermore, these experiments show that after sensitization the animals develop extensive disseminated leprous lesions throughout the body. The lesions have the same general gross appearance, except in color, as those of tuberculosis. Microscopically, however, the picture is distinctly different. Here the areas contain large numbers of mononucleated cells, in addition to the epithelioid type of cell, which are filled with viable leprosy bacilli.

Experiment X.—Four full grown white mice were inoculated on March 10, two intraperitoneally with approximately two million dead leprosy bacilli, and two subcutaneously with the same number of living organisms. The mice injected intraperitoneally with dead bacilli received, two weeks later, eight million viable lepra bacilli, one intraperitoneally and one subcutaneously. The animals were killed on April 12, and at autopsy they showed multiple lesions in the internal organs. The lesions were most extensive in the mouse which had received the intraperitoneal injections (figure 1). White mice appear to be more readily infected than any of the ordinary animals used in laboratory experiments, with the exception of the Japanese dancing mouse; in our experience, however, they, too, are more constantly infected by second inoculations.

SUMMARY AND DISCUSSION.

Repeated experiments have proven that few, if any, of the ordinary laboratory and domestic animals are immune against infection by *Bacillus lepræ*. As previously reported, the goat, horse, guinea pig, and many cold-blooded animals (Couret[5]) have been found susceptible to invasion by this organism.

Two factors are of great importance in effecting infection. In the first place, a sufficiently large number of organisms must be employed, and, what is still more important, second and subsequent inoculations are more liable to produce leprous lesions than are primary injections. Moderate doses used in the first inoculation of animals are comparatively harmless as regards their ability to induce lesions. Such preliminary doses, whether they consist of living or dead organisms, produce a condition of hypersensitiveness or allergy, which renders it possible by a second injection of viable bacilli to induce the development of a reactionary lesion. Lesions arising as the result of a second inoculation develop more rapidly, increase in size more quickly, and persist for a longer period than those taking place as the result of a single inoculation, even though very large doses are used. Moreover, the bacilli in these lesions are more liable to lead to metastasis and to a generalized infection. We regard the results of these experiments as having considerable bearing upon the development of the disease in human cases, since we find that it is chiefly among those living in prolonged intimate contact with leprous patients that leprosy develops.

The proper interpretation of these findings is difficult and becomes, apparently, more complex the longer they are studied. We are accustomed to similar phenomena of anaphylaxis or allergy with protein materials and with certain bacteria, especially the tubercle bacillus. It is not surprising that a specific allergy or altered reaction should take place in animals previously injected with leprosy bacilli, either alive or dead; why, however, lesions should develop in which the bacilli continue to grow in animals which had recovered from previous injections, or the serum of which showed bactericidal properties and contained other specific antibodies, is not so easily understood.

[5] Couret, *loc. cit.*

It is unnecessary in this paper to discuss the relative value of the different theories brought forward to explain the phenomenon of anaphylaxis. It must, nevertheless, be assumed that either as the result of the splitting of certain essentially non-toxic substances in the bacillus by specific ferment-like substances in the blood, which increase after a sensitizing dose, or by the joining of some body in the serum with certain substances in the bacilli, a toxic body is produced. Following the setting free or development of these injurious bodies, there results a cellular reaction, an area of local necrosis followed usually by the appearance of cells of the lymphoid and epithelioid type, and, especially if dead bacilli are used, by polymorphonuclear leucocytes. Such a reaction is usual in all allergic or anaphylactic conditions; but why the reaction should predispose to the more or less permanent development of parasitic powers on the part of the bacilli is not so plain. The idea has been expressed by us, as the result of observations based chiefly upon the study of the changes in the tissues of human leprosy, that the presence of bacilli within the large multinucleated cells that are characteristic of the lesion is the result of active multiplication of the bacilli in these cells rather than of phagocytosis by the cells, although it is possible that their original entrance may be the result of phagocytic action. If this be the case, it is possible that, through the cellular reaction plus the necrosis of certain of the fixed tissue cells, a pabulum of split protein products results upon which the bacilli feed, and in which they find protection from the antibodies present in the blood serum.

In our attempts to induce infection in various mammalian species with cultures of human leprosy, positive results have been obtained in almost every instance after the second injection of large numbers of the organism. In this manner, we have been successful in the production of lesions in the monkey, goat, horse, guinea pig, and mouse. The results of these experiments lead us to believe that the mechanism through which invasion and multiplication follows in these lower animals is similar to if not identical with that in man.

A careful study of the progress of the disease in man together with the behavior of the organism in the monkey and the goat

would suggest, at least, that the function of toxin production is, after all, of little use to the leprosy bacillus because it has seemingly acquired a highly parasitic existence; instead of which it is not only possible, but highly probable, that its defensive protection, whatever it may be, is enormously developed.

After the inoculation of animals with either dead or living bacilli, the production of specific antibodies is induced; these do not develop in any considerable amount and apparently show no marked tendency to increase upon repeated injections. The tests of the sera *in vitro* indicate that the antisubstances are produced chiefly by the first few injections. Either the existence of some specific bacterial protective body, or what seems more probable, the protection afforded by the host cells in which the bacilli become ensconsed accounts, we believe, for the difficulty of producing in animals an antiserum of high potency. The idea, too, that the bacilli do not multiply to any great extent until they have entered certain cells of the host has, in a large measure, been verified by the artificial cultivation experiments.

We know from our studies upon the biology of the organism that it will live for years in the most unfavorable conditions. This would suggest that during their sojourn in the tissue cells death as the result of autolysis rarely occurs, and as long as the cells can withstand the multiplication of the bacilli within and remain alive, so long are the bacilli protected from outside injurious influences, in consequence of which no disintegration of bacilli occurs to induce the production of immune bodies. On the other hand, death of the cells carrying the bacilli must occur from time to time, and this exposes large numbers of the organisms to the action of the body fluids, and, in consequence, to the condition of formation of specific antibodies.

EXPLANATION OF PLATES.

Plate 20.

Fig. 1. The gross appearance of leprous lesions in the peritoneal viscera of the white mouse. Note the distribution of nodules along the course of the mesentery vessels.

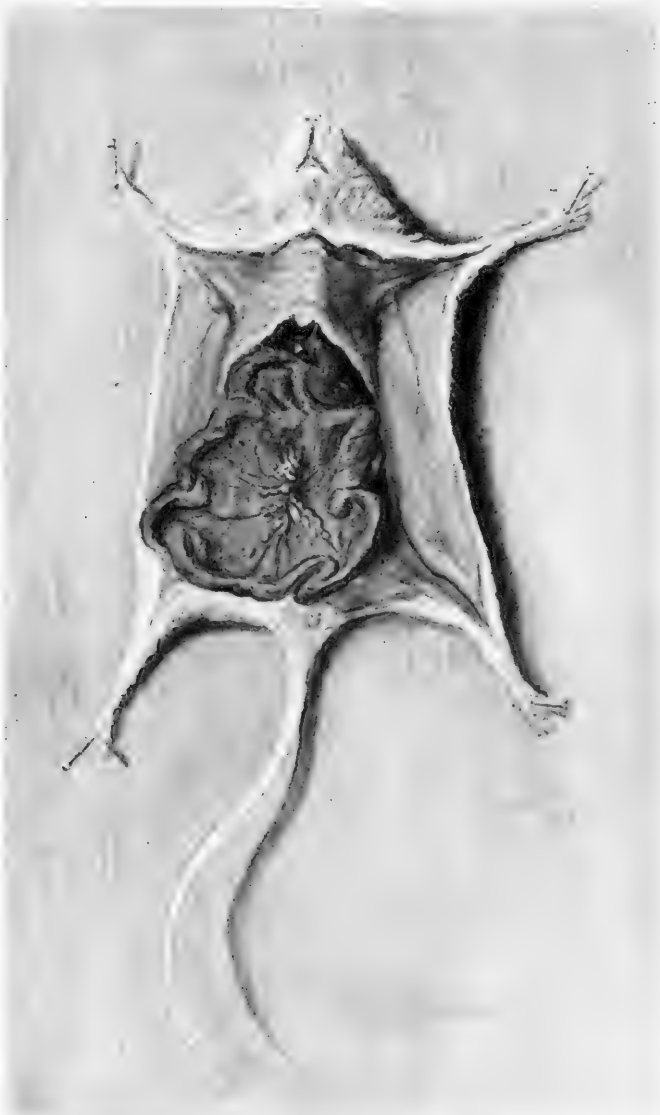

FIG. 1.

FIG. 2.

FIG. 3.

PLATE 21.

FIG. 2. A subcutaneous leprous nodule in a guinea pig, at the site of inoculation. The mass had attained a size of 2 cm. in diameter five days after the second injection of a pure culture of *B. lepræ*.

FIG. 3. A fatal infection with *B. lepræ* sixteen days after the second intraperitoneal injection. Note the extensive involvement of the liver, omentum, and under surface of the diaphragm. Note also the nodules in the pericardium and old fibrous adhesions attaching the organ to the sternum.

FURTHER OBSERVATIONS ON CERTAIN FEATURES OF EXPERIMENTAL SYPHILIS AND YAWS IN THE RABBIT.*

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INTRODUCTION.

The unique progress made during the last six years in our knowledge of the etiology, diagnosis, and treatment of syphilis has brought the present day study of this almost universally distributed disease into the first rank of scientific interest and practical importance; and students of tropical medicine are doubly concerned because this progress has, by analogy and by actual proof, been broadened to include the tropical group relative of syphilis, yaws. Much of this advance has been due to animal experimentation, and I desire at this time to present some observations of my own made in this field of work, relating to chemotherapy, to serum reactions, and to immunity.

While experiments on various species of monkeys have served to give us a great deal of information about syphilis and yaws, the rabbit has come to be the animal of choice for certain kinds of work not only because it is easy to obtain and to handle, but also on account of the nature of the lesions produced and the reactions of the host. It is interesting in this connection to note that, historically, the successful transmission of syphilis to rabbits antedates that to monkeys; for Haensell (1), after infecting rabbits in the anterior chamber of the eye with tubercular material, in 1881 undoubtedly produced a genuine syphilitic keratitis in rabbits, and transferred

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the virus to a second generation; little attention, however, was paid to his work because the specific nature of the lesions could not be demonstrated. Since the discovery of *Treponema pallidum,* four distinct kinds of lesions in the rabbit are recognized: first, corneal syphilis, which was definitely demonstrated by Bertarelli (2) in 1906; second, a syphiloma of the testicle, established by Parodi (3) in 1907 and worked out by Uhlenhuth and Mulzer (4) in 1909; third, a chancre of the scrotum demonstrated by Hoffmann, Löhe, and Mulzer (5), and Ossola and Truffi (6) in 1908, which appears either as an induration in and under the skin that ulcerates and forms a typical chancre, or as an induration of the tunica and subcutaneous tissue which does not extend to the exterior; and fourth, a septicemia, with generalized lesions in the skin and mucous membranes, especially of the nose, which has been produced, in the present year, by Uhlenhuth and Mulzer (7) by intracardial injections in very young animals. Even after the ocular and testicular inoculations, spirochætæ have occasionally been demonstrated in the glands, spleen, and liver, but secondary lesions are very rare, except those of the uninoculated eye or testicle.

In 1910, I (8) succeeded in producing a lesion of the testicle in rabbits with the treponema of yaws, and since then I have produced lesions of the scrotum, as is explained below.

The lesions of the eye are especially adapted for histological study on account of the simple structure of the cornea, and they also furnish an abundance of organisms in pure culture for various purposes. About 50 per cent. of these inoculations are successful. The lesions of the scrotum, as Hata (9) maintains, are, on the other hand, best adapted for investigation of the action of drugs, because the chancres are rich in treponema and are more accessible for examination than lesions of the eye or testicle. Here again only a certain percentage of the inoculations are successful. For the preservation of the strain in a living condition, the lesion of the testicle is most suitable, as almost 100 per cent. of inoculations are positive. I have also found this lesion best for the study of serum reactions and certain problems of immunity, because the lesions of the eye and of the scrotum are relatively superficial and hence seem not to set up such pronounced reactions in the host as do the testi-

cular lesions. It seems possible that all the local forms of inoculation will be superseded for general study, if Uhlenhuth and Mulzer are successful in developing further their general infections.

MATERIAL.

Syphilis.—I have been working with three strains of the spirochæta of syphilis and one of yaws; one strain of the syphilitic organism and the one of yaws are in the 15th transfer.[1] The other two strains of the syphilitic spirochæta are in the 3d and 6th transfers respectively. All three syphilitic strains were started in the testicle from human mucous patches. Little difficulty is encountered in making the first transfer if a suction apparatus is used on a mucous patch so that a considerable amount of infected serum is obtained for injection. Only two animals died of sputum septicemia, and no marked secondary infections occurred. The first strains of the treponema of syphilis and yaws yielded only 50 per cent. of "takes" in the first three passages, but the later passages of these strains and the early ones of the other syphilitic strains gave practically 100 per cent. of infections. A large quantity of material was employed for injection and large rabbits with well developed organs were selected for inoculation. There appears to be choice in rabbits in regard to color. The general adaptability, in my experience, was: white, grey, brown, and black. In Germany, the silver grey rabbit is chosen. The incubation period of the first transfers averaged 45 days; while in the 9th to the 13th transfers, it averaged 35 days, when fluid was used for injection. Hence the spirochætæ on successive transfers grew somewhat more rapidly; but no difference was noted in the size or duration between the lesions of the early and the later transfers. If a bit of tissue is introduced in a trocar, the incubation period is about 10 days shorter. The incubation period for the entire series of the first strain in 46 animals averaged 34 days. The lesion of the testicle, which is a myxomatous tumor, occurs either in the epididymis or testicle and varies in size from that of a pea to that of an olive. Small lesions occasionally occur which disappear in a short time, but well developed lesions last several weeks and are rich in spirochætæ.

The production of chancres is more uncertain and, as Hata has pointed out, in order to secure reliable results, many animals must be inoculated. The direct method consists in inserting a piece of infected tissue under the skin of the scrotum; many of these inoculations fail or begin to develop and then retrogress. Often, however, in inoculating the testicles some spirochætæ are caught in the scrotum and produce excellent lesions; at times a combination of the testicular lesion with lesions of the tunica and an ulcerating chancre is produced. Out of 13 chancres in my series, 11 followed inoculation of the testicle and only 2 resulted from 5 direct inoculations of the scrotum. The incubation period for the chancres averaged 43 days. Five inoculations of the anterior chamber of the eye failed to produce lesions.

Yaws.—The incubation period, with inoculations of fluid, of lesions of the testicle, caused by *Treponema pertenue,* for the

[1] An account of these strains was given in the *Jour. Exper. Med.,* 1910, xii, 616.

animals in the 1st transfer was 41 days, and for the last animals in the 9th to the 14th transfers it was 29 days. In tissue inoculation, the period averaged 23.5 days. Seven animals gave very short periods, the average being 13 days. For the entire series of 52 animals, the incubation period was 26 days, or 8 days shorter than that of the series of animals infected with the pallidum. No marked morphological differences have been noted in the nature of the lesions of the testicle in syphilis and yaws.

At first, I thought that *Treponema pertenue* would not produce lesions of the scrotum, but, at the suggestion of Hata, I inoculated 7 animals under the skin of the scrotum with testicular tissue in the 5th transfer. In 6 days, 2 animals developed hard nodules which enlarged to typical chancres; the other 5 failed to show lesions. Since then, 8 chancres have developed after inoculation of the testicle. The incubation period for the 10 chancre-like lesions averaged 23.5 days—a period less than half that in syphilis. In contrast to the differences seen in yaws and syphilitic lesions of the monkey's eyebrow, there is practically no difference in the lesions which are produced in the rabbit's scrotum by pallidum and pertenue, except as regards the incubation period. In explanation of this fact it may be suggested that the skin of a monkey's eyebrow, for example, differs from that of a rabbit's scrotum and is more nearly related to the human integument. Four inoculations of the anterior chamber of the eye have not resulted in infections.

CHEMOTHERAPY.

Salvarsan.—Hata, working with scrotal chancres, has shown that out of a large number of synthetic arsenic preparations, arsenobenzol has the greatest spirillicidal action, the ratio C/T being 1 to 10. A single intravenous dose of 0.01 gram per kilo permanently cured every lesion. Working with testicular lesions of the treponema of syphilis and yaws, I (10) have found that salvarsan destroys the treponema of yaws in a smaller dose (table I). Four rabbits infected with *Treponema pertenue* were permanently cured by a dose of 0.0045 gram per kilo, while out of 5 rabbits infected with pallidum and treated with a similar dose, 3 relapsed in 31, 36,

and 49 days, respectively. A smaller dose than 0.0045 gram per kilo, however, failed to kill the pertenue.

TABLE I.

Comparative Effect of the Same Dose of Salvarsan on the Lesions of Syphilis and of Yaws in the Rabbit.

Syphilis.	Condition.	Spirochætæ.	Dose per kilo in gms.	Remarks.
B	Large nodule, left testicle. Small nodule, right testicle.	+ +	May 9, 1910. 0.0045, intramuscular.	Lesions healed; relapse in left testicle in 31 days.
W	Small nodule, left testicle.	+	May 9, 1910. 0.0045, subcutaneous.	Lesion healed; relapse in 36 days.
U	Large nodule, left testicle.	+	May 9, 1910. 0.0045, intravenous.	Lesion healed; relapse in 49 days.
V	Small nodule, left testicle.	+	May 9, 1910. 0.0045, intramuscular.	Lesion healed; castrated 24th day; negative.
S	Large nodule, left testicle.	+	May 9, 1910. 0.0045, intravenous.	Lesion healed; castrated 9th day; negative.
Yaws.				
15	Large nodule, right testicle.	+	May 9, 1910. 0.0045, intramuscular.	Lesion healed; castrated 19th day; negative.
17	Small nodule, left testicle.	+	May 9, 1910. 0.0045, subcutaneous.	Lesion healed; no relapse in 66 days.
21	Large nodule, right testicle.	+	May 24, 1910. 0.0045, intramuscular.	Lesion healed; castrated 24th day; negative.
1	Large nodule, right testicle.	+	May 9, 1910. 0.0045, intravenous.	Lesion healed; no relapse in 38 days.
9 A 9	Large nodule, right testicle.	+	Aug. 8, 1910. 0.0025, intravenous.	No effect.
9 A 11	Small nodule, left testicle.	+	Aug. 10, 1910. 0.003, intravenous.	No effect.
5^{32}	Large chancre, both testicles.	+	Aug. 8, 1910. 0.003, intravenous.	No effect.
9 A 2	Large nodule, right testicle.	+	Aug. 10, 1910. 0.0035, intravenous.	No effect.
9 A 5	Large nodule, left testicle.	+	Aug. 8, 1910. 0.0075, intravenous.	Lesion healed.
9 A 8	Large nodule, left testicle.	+	Aug. 8, 1910. 0.0075, intravenous.	Lesion healed.

These differences seem to apply equally to man, as several observers in the tropics have been able to cure yaws with doses of salvarsan which would certainly have been followed by many relapses in syphilis. Both Strong (11) and Castellani (12) have treated a considerable number of adults with doses of 0.3 to 0.4 gram with but one one or two recurrences in a number of months.

This is an interesting fact and accords with the general conception that yaws is a localized infection of the skin rather than a disease in which the treponema can gain a permanent foothold in almost every tissue of the body. It is impossible to say, until cultures of the treponema have been obtained, whether the greater resistance of pallidum to treatment is due to some resistance of the organism itself or to a mechanical reason, such as an ability on the part of the organism to penetrate further into places where the drug does not circulate so freely.

The patient from whom the pertenua were obtained was finally cured by salvarsan. He suffered from three separate series of eruptions. The third one was treated without effect by atoxyl; under potassium iodid, the skin lesions healed rapidly and he gained in weight but still complained of joint-pains and showed a positive Wassermann reaction which he had failed to show earlier. In November, 1910, he was sent to the Walter Reed Hospital, in Washington, D. C., and on November 3 he was given 0.5 gram of salvarsan intramuscularly. His pains ceased in a few days; the serum reaction became negative in 16 days and has remained so.

The question of "arsenic fast" spirochætæ became an important one after the introduction of salvarsan. For the study of this question, I selected a soldier in the secondary stage of syphilis, who had received a subcutaneous injection of salvarsan. Slight improvement of the mucous patches had occurred followed by relapse. I inoculated 3 rabbits with serum taken from one of the mucous patches 24 days after treatment, and all 3 became infected. One was given 0.005 gram (one half the sterilizing dose) of salvarsan. The spirochætæ were active and numerous after 48 hours; the dose was then repeated and the spirochætæ and lesions disappeared, but recurred 16 days later. The strain was then transferred to 3 other rabbits and in these was promptly killed by doses of 0.005 and 0.01 gram per kilo. Hence, after four exposures, no "arsenic-fast" properties could be demonstrated. It is possible that by repeating smaller doses some resistance might be developed; but the aim of the experiments was to approximate clinical conditions as closely as possible. It seems feasible, therefore, to repeat the drug several times with good effect and without danger of producing resistant strains.

One animal only has shown a relapse after a dose of 0.01 gram per kilo, and this animal had previously received 0.025 gram of sodium cacodylate. A second relapse occurred after 0.01 gram per kilo, but the infection was finally entirely abolished by 0.02 gram per kilo. This case might be cited as an example of resistance to arsenic, but as it was only one out of a number, the relapse may equally well be explained by assuming that a few spirochætæ were hidden in the tissues away from the action of the drug.

Sodium Cacodylate.—Among arsenical preparations recently tried against syphilis, sodium cacodylate has had some vogue in this country. I (13) tested this drug thoroughly in rabbit syphilis and found that the ratio C/T = 1/1; that is, that the drug had no parasitotropic property. Alston (14) has recently tried the drug on yaws in Trinidad and says, "I injected four cases with cacodylate of soda for comparison, and see nothing to compare with the experiments with '606' and the serum." Neisser tested the preventive and curative properties of sodium cacodylate on monkeys infected with syphilis and concludes his account by quoting Ehrlich who said, "The preparation is indeed non-toxic but it is also therapeutically worthless."

Mercury.—As soon as exact methods were possible for the study of the action of drugs on the spirochætæ, a great deal of interest naturally arose concerning the exact action of mercury and potassium iodid. Various opinions have been held concerning the action of these drugs, but the consensus of opinion has been that mercury is a specific and that potassium iodid is a symptomatic remedy. A good deal of work has been done with repeated doses of the drugs in monkeys, and in rabbits with corneal infections; and, while mercury has been proved to be a specific in large doses, opinion is divided about potassium iodid (15). I have worked chiefly with single intravenous injections of the bichlorid and salicylate of mercury in order, if possible, to obtain a better comparison with salvarsan. I have found under these conditions that no action can be demonstrated for mercury much short of the fatal dose. The tolerated dose for mercuric chlorid is 0.001 gram per kilo (table II), but no definite spirillicidal action is demonstrable with this dose; if, however, the dose is raised to 0.003 gram the spirochætæ

TABLE II.

Tolerated Dose of Mercuric Chlorid; One Intravenous Injection.

Rabbit.	Weight in gms.	Dose per kilo in gms.	Remarks.
1	2,590	0.001	No effect.
2	2,560	0.002	Died in 11 days; nephritis.
3	2,420	0.003	Died in 11 days; nephritis.
4	2,060	0.003	Died in 5 days; nephritis.
5	2,150	0.004	Died in 9 days; nephritis.
6	2,390	0.005	Died in 4 days; nephritis.
7	1,950	0.005	Died in 1 day; nephritis.
8	1,810	0.005	Died immediately; convulsions.
9	2,250	0.01	Died immediately; convulsions.

are destroyed in 48 hours (table III), but the animal eventually dies of nephritis.

The same is true of the salicylate; the tolerated dose of 0.005 gram per kilo has no certain curative effect, but a dose of 0.0075 or

TABLE III.

Effect of Mercuric Chlorid on the Spirochæta of Syphilis and of Yaws; One Intravenous Injection.

Syphilis.	Dose per kilo in gms.	Day.								Remarks.
		Spirochætæ.	1	2	3	4	5	6	7	
XXXVII, Ch.	Feb. 27, 0.0005	+	+	+	+	+			+	No effect on spirochætæ.
	Mar. 6, 0.001	+	+		+			—		Healed on 20th day. Spirochætæ — 12th and 20th days.
XLIII, Ch.	Apr. 4, 0.003	+	+	—	—	—		—		Spirochætæ killed; died on 11th day.
2/10, T.	Apr. 7, 0.003	+	—		—					Spirochætæ killed; died on 50th day.
Yaws.										
40, T.	Feb. 27, 0.0005	+	+	+	+	+			+	No effect on spirochætæ.
	Mar. 6, 0.001	+	+		+			+		No effect on spirochætæ.
45, Ch.	Feb. 27, 0.0005	+		+		+			+	No effect on spirochætæ.
	Mar. 6, 0.001	+	+		+					No effect on spirochætæ.
	Mar. 20, 0.0015	+	+		+				+	No effect on spirochætæ.

+ = Spirochætæ present, active.
± = Spirochætæ present, motionless.
— = Spirochætæ not found.
F. = Spirochætæ few.
Ch. = Chancre.
T. = Testicular lesion.

of 0.01 gram will destroy the parasites promptly, although the animal usually dies later of nephritis (tables IV and V). In these

TABLE IV.

Tolerated Dose of Mercuric Salicylate; One Intravenous Injection.

Rabbit.	Weight.	Dose per kilo in gms.	Remarks.
1	2,230	0.005	No effect.
2	2,130	0.005	Died in 15 days; nephritis.
3	2,430	0.0075	No effect.
4	2,000	0.01	Died in 13 days; nephritis.
5	2,055	0.01	Died in 10 days; nephritis.
6	1,930	0.01	Died in 7 days; nephritis.
7	1,960	0.01	Died in 4 days; nephritis.
8	1,980	0.01	Died in 3 days; nephritis.
9	2,030	0.01	Died in 3 days; nephritis.
10	2,240	0.025	Died in 2 days; nephritis.
11	2,380	0.05	Died immediately.

TABLE V.

Effect of Mercuric Salicylate on the Spirochæta of Syphilis and of Yaws.

Syphilis.	Dose per kilo in gms.	Spirochætæ.	Day.							Remarks.
			1	2	3	4	5	6	7	
2/7, T.	Feb. 20, 0.005	+	+	+	F+	F+			+	No cure; intramuscular in oil.
2/8, Ch.	Apr. 7, 0.01	+	+±		+±	—			—	Spirochætæ killed; died on 9th day; intravenous injection.
1/44, T.	Apr. 7, 0.01	+	—		—					Spirochætæ killed; died on 3d day; intravenous injection.
Yaws.										
48, Ch.	Mar. 7, 0.005	+	+	+			+			No effect on spirochætæ; intravenous injection.
	Mar. 20, 0.0075	+	+		—				—	Spirochætæ killed; intravenous injection.
49, T.	Mar. 1, 0.005	+	+	—			—			Spirochætæ killed; died on 14th day; intravenous injection.
51, Ch.	Apr. 4, 0.01	+	+F	—	—	—		—		Spirochætæ killed; intravenous injection.
1/33, T.	Mar. 31, 0.01	+	+F		—	—				Spirochætæ killed; died on 12th day; intravenous injection.

experiments, the insoluble form of mercury salicylate was mixed with equal parts of potassium carbonate to dissolve it. Rabbits are, of course, subject to nephritis naturally, but the lesions found after injection of mercury were so definite and the mortality was so great that no doubt was left as to the cause of the condition. In animals dying early, the kidneys were pale and swollen and showed an acute

necrosis especially of the collecting tubules. In the later stages, a high degree of calcification had set in—enough to nick the edge of the section knife.[2] The doses used would represent 1 to 3 grams of mercuric chlorid and 5 to 10 grams of mercuric salicylate for adults of 70 kilos.

It is evident from these results, as well as those of other observers, that mercury has some selective action for spirochætæ; but it is evident that the margin between the parasitotropic action and the organotropic action is not nearly as great as it is with salvarsan, from which one can understand why it is necessary to continue mercury so long clinically to get beneficial results. It is also evident that the intensive treatment with mercury is indicated in order to affect the spirochætæ promptly and that at the same time the effects on the kidneys must be watched constantly.

Potassium Iodid.—The tolerated dose of potassium iodid is shown in table VI and is about 0.03 gram per kilo. If the dose is

TABLE VI.

Tolerated Dose of Potassium Iodid; One Intravenous Injection.

Rabbit.	Weight.	Dose per kilo in gms.	Remarks.
1	2,260	0.01	No apparent effect.
2	1,980	0.025	No apparent effect.'
3	1,960	0.03	No apparent effect.
4	2,880	0.05	No apparent effect.
5	2,000	0.05	Convulsion ; died immediately.
6	2,000	0.1	Convulsion ; died immediately.
7	2,580	0.2	Convulsion ; died in 10 days.
8	2,500	0.5	Convulsion ; died immediately.

raised above this amount the rabbit dies in most cases immediately. Table VII shows the action on the spirochætæ; and it is evident that no definite effect is produced by this dose. That potassium iodid has some action on the spirochætæ is evident, however, from animal XXXIV, in which there was a definite curative action; the spirochætæ became reduced in number and the lesion healed in ten days. The dose, however, was 1 gram per kilo injected into the muscles which became necrotic and atrophied. In animal 52, 0.5

[2] This is the characteristic renal lesion of the rabbit following mercury intoxication.

TABLE VII.

Effect of Potassium Iodid on the Spirochæta of Syphilis and of Yaws.

Syphilis.	Dose per kilo in gms.	Spirochætæ.	Day. 1	2	3	4	5	6	7	8	Remarks.
2/8, Ch.	Mar. 22, 0.01	+	+				+		+		No effect; intravenous injection.
	Mar. 29, 0.03	+	+	+		+		+			No effect; intravenous injection.
	Apr. 4, 0.03	+	+	+	+						No effect; intravenous injection.
XXXIV, Ch.	Jan. 31, 1.0	+	+	F+	F+	±		F+		—	Healed in 10 days; intramuscular injection.
Yaws.											
50, T.	Apr. 7, .03	+	—		+	+	±		—		Healed in 10 days; intravenous injection.
52, T.	May 12, .5	+	+		±	+				+	No effect after 10 days; subcutaneous injection.

gram given subcutaneously produced an extensive necrosis, after which it was thought unprofitable to carry the study further. Neisser (16) has been able to prevent and to cure infections in monkeys with doses of 7 to 8 grams, but no such dose could be tolerated for man; hence it is doubtful if potassium iodid can be used with advantage as a spirillicide in the early stages of syphilis, and its chief value, according to the present usage, seems to be to aid absorption in yaws and in the later stages of syphilis. The parasitotropic action of these drugs is so small that it is impossible to determine any differences for the two organisms.

SERUM REACTIONS.

I have found that the testicular infection in the rabbit serves well for observations on the complement fixation test in syphilis and in yaws. The reaction comes on in about 10 to 14 days after the lesion appears, increases in intensity, and disappears as the lesion heals spontaneously or as the result of treatment, or after it is removed by castration. Several authors, namely, Schilling (17) and Browning (18), working with trypanosome infections, have held that rabbits are unsuitable for observations of this kind because they normally give reactions; but Captain Craig, of the

Medical Corps, and I, after many observations, have as yet to find a positive reaction in 50 normal rabbits. Schilling states that he found 7 positive out of 12 normal rabbits; but this reaction has been ascribed to coccidia or some other parasite as well as to some peculiarity of the normal rabbit. Uhlenhuth and Mulzer, however, found 16 normal animals negative. At all events, in our hands, the results have been entirely uniform with an alcoholic extract of syphilitic liver as antigen and 0.05 to 0.2 cubic centimeters of inactivated serum.

In 20 syphilitic rabbits, the reaction became positive in an average of 9.4 days after the lesion appeared in the testicle; in 8 of the animals, it was negative on an average at 6.5 days. Chancres of the scrotum, however, failed to give good reactions even after 3 to 4 weeks. The same negative result has been reported for lesions of the eye. In order to secure a good reaction, it is apparently necessary that the lesion should be in an organ well supplied with blood rather than in the relatively avascular skin or cornea.

In the case of infections with the treponema of yaws, the reaction became positive in 10 animals in an average of 13.2 days after the lesion appeared; in 5 of the animals it was negative in an average of 11.4 days after the lesion appeared. The reaction has remained positive up to one month, depending strictly on the course of the infection.

An interesting finding depends on the fact, already mentioned, that the spirochætæ travel to the opposite testicle, probably by way of the spermatic vessels. In these cases, after the inoculated testicle has been removed, the serum reaction may disappear only to reappear when the uninoculated testicle becomes affected. On extirpation of the second testicle, the reaction disappears permanently, as is shown in chart 1. This finding speaks against the occurrence of definite lesions in the internal organs, even assuming that the spirochætæ circulate to a certain extent in the blood.

The action of salvarsan is shown in charts 3 and 4 for a typical infection with both syphilis and yaws. Two days after the injection, the serum became negative, then again positive in 2 days, and then permanently negative. This secondary rise may be correlated with the occasional finding of a negative reaction which be-

comes positive after the injection of salvarsan, and is probably due to a reaction of the tissues to bodies set free on the disintegration of the destroyed treponema.

Captain Craig has kindly furnished me with table VIII, which

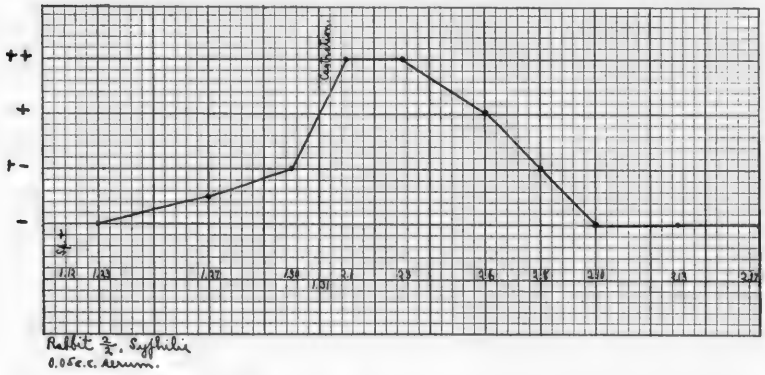

CHART 1.—Rabbit 2/2; right testicle inoculated October 11, 1910; nodule with spirochætæ, November 28; 48 days. Right testicle castrated December 6. Left testicle, nodule with spirochætæ, January 12, 1911.

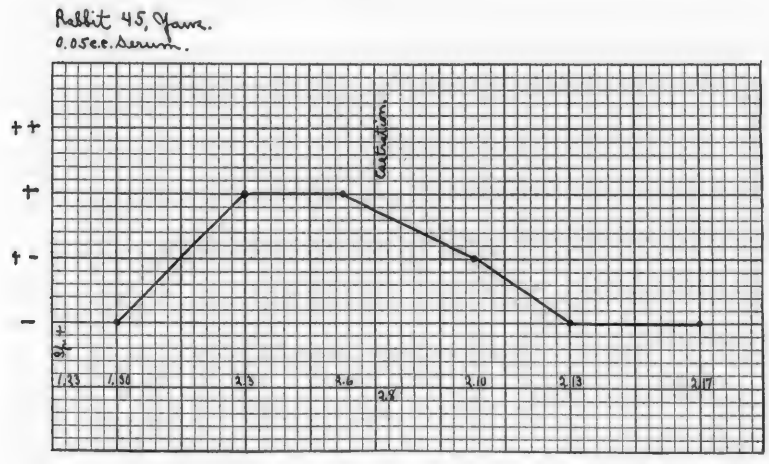

CHART 2. Rabbit 45; inoculated December 23, 1911; nodule with spirochætæ, January 23, 1911; 31 days.

shows the effect of treatment in the case of two of the animals with which we have been working.

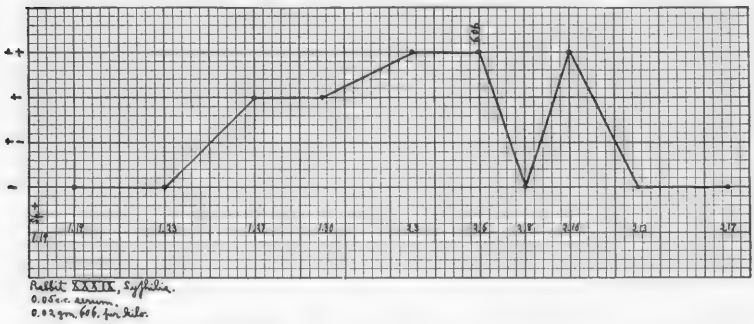

CHART 3.—Rabbit XXXIX; inoculated December 23, 1910; nodule with spirochætæ, January 19, 1911; 27 days.

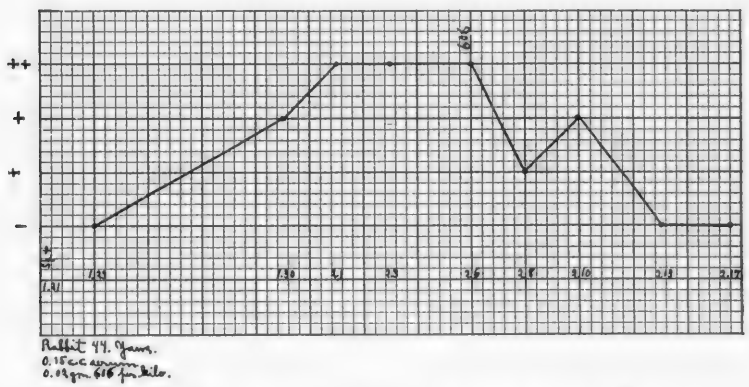

CHART 4.—Rabbit 44; inoculated December 23, 1910; nodule with spirochætæ, January 21, 1911; 29 days.

Captain Craig and I have, however, been unable to make any use of specific antigens made from rabbits' testicles (table IX). We made antigens from normal testicles and from those infected with pallidum and pertenue. One part of infected tissue was ground up and shaken with 10 parts of absolute alcohol and after titration the following quantities were used: yaws and syphilitic testicle antigen, 0.06 c.c.; normal testicle antigen, 0.08 c.c.; stock antigen, 0.02 c.c.; and inactivated serum, 0.1 c.c. Both normal men and animals gave positive reactions with the normal testicle antigen and with the specific antigens, and infected men and

TABLE VIII.

Results of Complement Fixation Test before and after Treatment with Salvarsan in Rabbit No. 39, Infected with Lues.

Amount of serum in c.c.	Date of tests.								Remarks.
	Jan. 23	27	30	Feb. 6	8	10	13	18	
0.05	—	+	+	++	—	++	0	—	Spirochætæ found in testicular lesion, Jan. 19, 1911. 0.02 gm. salvarsan per kilo administered intravenously, Feb. 6, 1911.
0.10	—	++	++	++	+—	++	—	—	
0.15	0	++	++	++	+—	++	0	—	
0.20	++	0	++	++	+—	++	—	—	

Results of Complement Fixation Tests before and after Treatment with Salvarsan in Rabbit No. 44, Infected with Yaws.

Amount of serum in c.c.	Date of tests.								Remarks.
	Jan. 23	30	Feb. 2	6	8	10	13	17	
0.05	—	—	++	++	—	—	—	—	Spirochætæ found in lesion, Jan. 21, 1911. 0.02 gm. salvarsan per kilo administered intravenously, Feb. 6, 1911.
0.10	—	+—	++	++	—	+—	—	—	
0.15	—	+	++	++	+—	+	—	—	
0.20	—	++	++	++	+—	++	—	—	

TABLE IX.

Results of the Use of Various Antigens and Various Sera.

	Stock antigen.	Rabbit normal testicle antigen.	Rabbit syphilitic testicle antigen.	Rabbit yaws testicle antigen.
1. Normal man.	—		+	+
2. Normal man.	—		+	+
3. Normal man.	—		+—	+
4. Normal man.	—	+—	—	+—
1. Syphilitic man.	++	++	++	
2. Syphilitic man.	++	—		
3. Syphilitic man.	++	—	—	++
1. Yaws patient Oct. 24.	+		+—	++
1. Yaws patient Nov. 3.	+	+—	+—	—
1. Rabbit syphilis.	+	+		
2. Rabbit syphilis.	+	+—	—	—
3. Rabbit syphilis.	+	—	++	+
1. Rabbit yaws.	++	++	++	++
1. Normal rabbit.	—		+	+
2. Normal rabbit.	—	+		+
3. Normal rabbit.	—	+	+	+
4. Normal rabbit.	—	+—		—
5. Normal rabbit.	—	+	—	+—

animals reacted very irregularly, while our results with the stock antigen of syphilitic liver were constantly uniform. Our experience agrees with that of Baermann and Wetter, who have reported that they could not demonstrate any specific nature in antigens made from yaws nodules. Castellani (19) has reported positive results of this kind on monkeys, but we have never been able to secure a sufficiently strong reaction in monkeys for such a test and do not

believe that the complement fixation test can be used to advantage in this way, at least not until cultures of the pertenue are obtained.

In connection with the finding of a positive reaction in yaws rabbits, Baermann and Wetter's (20) results in Java are of interest. Among fresh cases with rich eruption they found 100 per cent. positive; among fresh cases with few yaws, 80 per cent.; and in treated cases 50 per cent. In some cases the reaction became negative after 2 or 3 injections of 1.5 grams of mercuric salicylate, and in none except the fresh cases was the reaction strong. My yaws patient failed to give a reaction after the second and third eruptions; and in 4 monkeys no reaction has been obtained up to 5 months after infection.

IMMUNITY.

Until recently, it has been generally held that an attack of syphilis conferred immunity, and cases of reinfection were cited as unusual. But we have come to realize, especially since the work of Neisser on monkeys, that there is no true immunity in syphilis. If an animal, previously infected, resists reinoculation, the animal is not strictly immune, but does not become reinfected merely because it still has the disease. When the disease is entirely abolished, either naturally or by treatment, reinfection occurs regularly. I have seen several interesting occurrences bearing on this point in the case of both syphilis and yaws. Rabbits can be infected in both testicles if inoculated at the same time or within one to two weeks of each other; but if the second testicle is inoculated after the appearance of a lesion in the first testicle, no take occurs while the lesion of the first testicle is still active. If, however, the lesion of the first testicle retrogresses or if the testicle is extirpated, spontaneous lesions occurred in the second testicle in 5 out of 6 instances (table X).

TABLE X.

Spontaneous Infections of the Second Testicle after Castration or Regression of the Lesion of the First Testicle.

Rabbit.	Inoculated.	Spirochætæ +.	Days.	Regression or castration.	Spirochætæ + or —.	Days after first lesion.	Days after castration or regression.
Syphilis.							
G.	R., Jan. 31, 1910	Mar. 17	45	Cas., Mar. 19	+L., Apr. 22	36	34
XXI	R., Oct. 4, 1910	Oct. 31	27	Reg., Nov. 9	+L., Nov. 21	21	12
XLVI	R., Mar. 4, 1911	Mar. 20	16	Reg., May 4	+L., May 15	56	11
Yaws.							
9	R., Mar. 29, 1910	Apr. 22	24	Cas., Apr. 27	+L., May 27	35	30
40	R., Nov. 14, 1910	Dec. 27	43	Cas., Jan. 14	+L., Feb. 27	62	44
26	R., May 31, 1910	June 14	14	Cas., June 15	—L., Aug. 30	75	76

L. = left. R. = right.

When the second testicle was inoculated after removal of the first infected testicle, a take resulted in 5 out of 6 cases. When the second testicle was inoculated after the first had developed a lesion, but without castration, a take occurred in 3 out of 6 cases; and in these a regression of the first lesion was going on. As long as the first lesion persisted actively, no take occurred in the second testicle (table XI).

TABLE XI.

Reinfections of the Second Testicle after Castration or Regression of the Lesion in the First Testicle.

Rabbit.	Inoculated.	Spirochætæ +.	Days.	Regression or castration.	Inoculation.	Spirochætæ + or —.	Days.	Days after castration or regression.
Syphilis.								
II	R., Apr. 18	May 25	37	Cas., June 14	L., June 14	+ July 28	38	38
3/5	R., Nov. 11	Dec. 27	46	Cas., Jan. 17	L., Jan. 17	+ Apr. 10	83	83
VIII	L., May 20	June 14	25	Reg., June 28	R., June 15	+ Aug. 29	76	63
II	L., Apr. 18	June 14	57	Reg., July 22	R., June 15	— Aug. 29	74	38
IX	L., May 28	June 20	23	Reg., June 28	R., June 23	— Aug. 29	66	58
Yaws.								
5	R., May 17	May 27	10	Cas., June 14	L., June 14	+ June 28	14	14
10	R., Mar. 29	Apr. 18	20	Cas., May 10	L., May 10	+ June 20	41	41
24	R., May 20	May 27	7	Cas., May 31	L., June 14	+ July 14	30	44
6	R., Feb. 25	Apr. 8	42	Cas., Apr. 14	L., Apr. 22	— June 15	54	62
A 1	R., Mar. 10	Apr. 22	43	Reg., May 13	L., Apr. 23	+ May 27	34	14
14	R., Mar. 31	Apr. 22	22	Reg., Apr. 29	L., Apr. 23	+ May 13	20	14
27	R., May 31	June 23	24	No reg	L., June 23	— Aug. 30	68	0

The same relations hold good when an infection is terminated by salvarsan (table XII). There is a resistance to reinfection for at

TABLE XII.

Reinfections after Treatment with Salvarsan.

Rabbit.	Inoculated.	Spirochætæ +.	Days.	Salvarsan intravenous per kilo in gms.	Days after lesion.	Inoculated.	Days after salvarsan.	Spirochætæ + or —.	Days.
Syphilis.									
XIX	R., Oct. 4, 1910	Oct. 24	20	Dec. 22, 0.01	59	L., Jan. 5, 1911	14	—Mar. 4	58
XX	R., Oct. 4, 1910	Nov. 1	27	Dec. 3, 0.01	32	L., Dec. 19	16	—Mar. 4	77
XXXV	L., Nov. 25, 1910	Dec. 19	24	Dec. 31, 0.01	12	R., Jan. 13, 1911	13	—Mar. 4	63
XXXVII	R., Dec. 10, 1910	Jan. 23, 1911	44	Mar. 29, 0.02	65	L., Apr. 11	13	—May 31	50
3/7	R., Jan. 17, 1911	Feb. 6	20	Feb. 9, 0.01	3	L., Mar. 29	48	—May 13	45
3/8	R., Jan. 17, 1911	Feb. 6	20	Feb. 14, 0.005	8	L., Mar. 29	43	+Apr. 14	16
XXXVIII	R., Jan. 17, 1911	Jan. 23,	31	Feb. 6, 0.02	14	L., Mar. 4	26	+Apr. 17	44
XXXV	L., Nov. 25, 1910	Dec. 19	24	Dec. 31, 0.01	12	R., Mar. 4	63	+Mar. 20	16

least two weeks after treatment, probably due to the transitory presence of antibodies, a condition that may be illustrated by the observed fact that suckling syphilitic babies are benefited if the infected mother is treated with salvarsan, although no arsenic can be demonstrated in the milk; and by the recent finding also, of Alston's, that yaws can be cured by injecting the serum of another case of yaws after treatment with salvarsan. Reinoculation with pallidum in rabbits was successful, however, on the 26th, 43d, and 63d days after treatment.

The results given above show that the spirochætæ are not confined to the testicle but that they migrate to other parts of the body and are able to produce lesions elsewhere if the conditions are favorable. The organisms must travel by the blood, as Neisser has been able to demonstrate them in the spleen and liver after inoculation of monkeys. It seems to me probable that they also travel by the spermatic vessels, because in every case of spontaneous infection the lesions have occurred in the epididymis minor or major, and these are the organs that the spirochætæ would first reach by the spermatic route. But while the organisms are disseminated over the body to a certain extent, they produce lesions only in the opposite testicle, or very rarely in the eye. Testicular lesions, but no others, have been produced by Uhlenhuth and Mulzer by intravenous inoculation; and the disappearance and reappearance of the serum reaction after castration also show that the organisms do not multiply except in the testicle.

The results given above demonstrate the inhibitory influence of a previous infection and the absence of immunity, and have on this account some bearing on human syphilis, as it is well known that reinfection with a chancre does not occur when the first chancre is well established; but several reinfections with chancres have already been reported shortly after the first chancre was cured by salvarsan.

It will be remembered that one of the arguments for the specific differences between yaws and syphilis rests on the immunity, or, more properly, the resistance experiments made by Neisser, Baermann, and Halberstädter (21). They showed that monkeys could not be reinfected with yaws or syphilis, but that a monkey with yaws would develop syphilis, and *vice versa*. At first I thought

that rabbits would be suitable for such experiments, but the phenomenon of the migration of the treponema complicated matters, because if a lesion occurred in the second testicle the organisms had to be identified, and I did not feel competent on morphological grounds alone to distinguish the organisms; and the only other way was to transfer them each time to monkeys,—a procedure which would have required a large number of monkeys. However, after salvarsan became available, one could treat infected rabbits with 0.01 to 0.02 grams per kilo, a sterilizing dose, and then inoculate the opposite testicle with each organism shortly after treatment.

Table XIII shows that of 4 animals previously infected with

TABLE XIII.

Cross Infections with Syphilis and Yaws after Treatment with Salvarsan.

Rabbit.	Inoculated syphilis.	Spirochætæ +.	Days.	Salvarsan per kilo in gms.	Days after lesion.	Inoculated yaws.	Days after salvarsan.	Spirochætæ + or —.	Days.
Syphilis—Yaws.									
XXXIII	R., Nov. 25, 1910	Dec. 12	17	Jan. 19, 1911. 0.01	38	L., Feb. 3	15	+Mar. 20	45
XXXVI	R., Nov. 25, 1910	Jan. 3, 1911	38	Jan. 19. 0.01	16	L., Feb. 3	15	+Mar. 27	52
3/4	R., Nov. 11, 1910	Jan. 3	53	Jan. 19. 0.01	16	L., Feb. 3	15	+Apr. 3	59
XXIX	R., Oct. 25, 1910	Dec. 27	63	Jan. 19. 0.01	23	L., Feb. 3	15	+Apr. 3	59

pallidum and then treated and reinoculated with pertenue on the 15th day after treatment all became infected, although the lesions were smaller than usual and the incubation period, 45 to 59 days, was longer than usual. As is shown in table XII, 4 animals similarly treated and reinoculated with pallidum on the 13th to the 16th days after treatment failed to develop lesions. Hence, in rabbits as well as in monkeys a difference can be demonstrated by inoculation experiments between the two diseases.

A similar set of experiments with animals infected with *Treponema pertenue* is under way and will be reported later.

To summarize: the following differences in experimental infection of yaws as compared with syphilis have been noted in the course of this work. The incubation period for testicular lesions is 8 days shorter; for skin lesions it is 20 days shorter. Lesions of yaws can be cured with smaller doses of salvarsan. The serum

reaction does not appear as promptly. Syphilitic rabbits treated with salvarsan can be infected with yaws 14 days after treatment, while reinfections with syphilis do not occur under the same conditions.

SUMMARY.

1. Lesions of the testicle and scrotum of the rabbit produced by the treponema of syphilis and of yaws afford material for the study of chemotherapy, of serum reactions, and of immunity.

2. On experimental and clinical grounds, it appears that yaws can be cured with a smaller dose of salvarsan than is necessary to cure syphilis.

3. The spirochætæ of syphilis do not become resistant after repeated treatments with salvarsan.

4. Sodium cacodylate has little effect on the treponema of either disease.

5. Mercury, in a single intravenous dose, has a spirillicidal effect, but the necessary dose usually causes the death of the animal later from nephritis.

6. Potassium iodid, in a single intravenous dose, has no spirillicidal effect.

7. The complement fixation reaction is closely correlated with the course of an infection, as is shown by its onset and its disappearance after castration or treatment.

8. Reinfection during the course of a testicular lesion does not occur, but it occurs after regression, treatment, or extirpation.

9. Specific differences between *Treponema pallidum* and *Treponema pertenue* can be demonstrated in infected animals treated with salvarsan and reinoculated.

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